DESIGN OF siRNA SEQUENCES AND *in vitro* EVALUATION AGAINST STRATEGIC TARGETS OF *nef* GENE OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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A Dios por todas las bendiciones recibidas
y por este nuevo logro alcanzado.
A mi familia por el amor y el apoyo brindado,
sin los cuales no hubiera sido posible culminar esta meta.

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LIST OF ABBREVIATIONS

aa	amino acid
AIDS	Acquired Immunodeficiency syndrome
bp	base pair
CCR5	C-C (beta) chemokine receptor 5
CxCR4	C-X-C (alpha) chemokine receptor 4
d2EGFP:	Destabilized Enhancer Green Fluorescence protein.
ds	double stranded
Env	Envelope protein
FACS	Fluorescence-Activated Cell sorter
Gag	Group specific antigen
GAPDH	Gliceraldehyde phosphate dehydrogenase
HIV	Human Immunodeficiency virus
HIV-1 R5	HIV-1, which uses the CCR5 receptor
HIV-1 X4	HIV-1, which uses the CXCR4 receptor
IN	Integrase
LTNP	Long term non-progressors
LTR	Long terminal repeat
MHC	Major Histocompatibility complex
mRNA	messenger-ribonucleic acid
Nef	negative factor
Pr	Protease viral
Rev	Regulator of virion expression
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT	Reverse transcriptase
shRNA	short hairpin RNA
siRNA	small interfering RNA

SIV	Simian Immunodeficiency virus
SIVsm	SIV derived from the sooty mangabey monkey
ss	single stranded
ssiRNA	synthetic small interfering RNA
TAR	Tat-responsive element
Tat	transactivator
TRBP	TAR RNA-binding protein
Vif	virion infectivity factor
Vpu	viral protein R

DESIGN OF siRNA SEQUENCES AND *in vitro* EVALUATION AGAINST STRATEGIC TARGETS OF THE *nef* GENE OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1*

AUTHORS: ALVAREZ-ROZO, Lenis; ARTEAGA, H José.**

KEY WORDS: HIV-1, *nef* gene, RNA interference, small interfering RNA sequence, fusion reporter protein Nef-dEGFP

ABSTRACT:

RNA interference (RNAi) is a conserved biological function of gene silencing mediated by double stranded small interfering RNA (siRNA) oligonucleotides, which cleavage only exact complementary mRNA. RNAi acts as an antiviral and also as a gene-expression regulation system. Several sequences of different HIV-1 genes have been efficiently targeted by artificial siRNA. However, given to the high mutation rate of the HIV-1 genome viral escape mutants are rapidly induced. Essential proteins for viral replication should work at a wide extent of mutated variants. Conversely, proteins conferring viral virulence, but not essential for replication, when targeted by siRNA, could induce low virulence escape mutants which are under lower selective pressure. Strategic selection of targets, i. e., sequences coding for highly conserved and functional domains, may attenuate this phenomenon or produce disable mutated HIV-1 strains.

Nef is a viral virulence protein, but not necessary for replication. In this work, four strategic siRNA targets were recognized for *nef* at the polypurine tract and myristoylation, Proline-rich motif and dimerization regions. Efficient siRNAs against them were identified from 11 shRNA sequences designed by hand after cloning them as shRNAs into a pol III plasmid and co-transfection in HelaT4 cells with a Nef-GFP reporter vector. Highly efficient shRNAs against the Proline-rich motif (2 with more than 95% of reporter expression inhibition), the Myristoylation signal (1 with 95%), the dimerization (2 whit 95%) and the Polypurine tract (1 with 85%) regions were identified.

Furthermore, plasmids expressing shRNA against HIV-1 generated by this approach may be better candidates for avoiding induction of escape mutants.

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*Thesis of grade of Master in Basic Biomedical Sciences

** Faculty of Health, Medicine School. Master in Basic Biomedical Sciences. Thesis supervisor.

DISEÑO DE SECUENCIAS siRNA Y EVALUACIÓN in vitro CONTRA BLANCOS ESTRATÉGICOS DEL GEN nef DEL VIRUS DE LA INMUNODEFICIENCIA HUMANA TIPO 1 *

AUTORES: ÁLVAREZ-ROZO, Lenis; ARTEAGA, H José.**

Palabras Clave: VIH-1, gen *nef*, RNA interferente, secuencias pequeñas de RNA interferente-siRNA, proteína de fusión reportera Nef-dEGFP

RESUMEN:

RNA interferente (RNAi) es una función biológica conservada de silenciamiento genético mediada por oligonucleótidos pequeños de RNA interferente que destruyen de manera específica RNA mensajeros complementarios. RNAi actúa como un sistema antiviral, así como un sistema de regulación de la expresión genética. Diferentes genes del VIH-1 han sido eficientemente silenciados mediante siRNAs. Sin embargo, mutantes que escapan a este efecto son rápidamente inducidos. Proteínas esenciales para la replicación viral deben trabajar en un amplio rango de mutantes. En caso contrario, proteínas que confieren virulencia, pero no son esenciales para la replicación, cuando son blancos de siRNA podrían inducir la aparición de mutantes de baja virulencia que están bajo una presión selectiva menor. Blancos estratégicos, como secuencias que codifican para dominios altamente conservados y funcionales, pueden atenuar este fenómeno o producir cepas no viables de VIH-1.

Nef es una proteína de virulencia, pero no es necesaria para la replicación. En este trabajo, cuatro blancos de siRNA fueron reconocidos para Nef en el tracto de polipurina, región de miristilación, motivo rico en prolinas y región de dimerización. Eficientes siRNAs contra estas regiones fueron identificadas de 11 secuencias de siRNA en forma de pinza (shRNA), las cuales fueron diseñadas manualmente, clonadas como shRNA en un plásmido Pol III y co-transfectadas en células HeLaT4 con un vector reportero que expresa la proteína de fusión Nef-dEGFP. Se identificaron shRNAs altamente eficientes contra la región rica en prolinas (2 secuencias con más del 95% de inhibición), la señal de miristilación (1 secuencia con 95% de inhibición), la región de dimerización (2 secuencias con 95% inhibición) y el Tracto de Polipurina (1 secuencia con 85% de inhibición).

Por lo tanto, plásmidos que expresan shRNAs contra VIH-1 generados mediante este método pueden ser mejores candidatos para evitar la inducción de mutantes que escapan al efecto de RNA interferente.

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INTRODUCTION

The Human Immunodeficiency Virus (HIV) is the etiologic agent of Acquired Immunodeficiency syndrome (AIDS). The HIV infection has became a pandemic of unprecedented dimensions affecting millions of people since the first clinical evidence of AIDS was reported. HIV/AIDS has spread to every country of the world, especially low- and middle- income countries. The HIV is a small virus but its pathogenesis is very complex resulting in the destruction of the immune system, notably CD4+ T cells at individual infected. Globally, an estimated 33 million people were living with HIV in 2007 and overall, 2.0 million died due to AIDS in the same year. Southern Africa continues to bear a disproportionate share of the global burden of HIV: 35% of HIV infections and 38% of AIDS deaths in 2007 occurred there.

Regarding the estimated number of people living with HIV in Latin America in 2007 was 1.7 million, and an estimated of 63000 people died of AIDS. HIV transmission in this region is occurring primarily among men who have sex with men, sex workers and injecting drug users. Colombia has a prevalence of 0.6%, including 170000 men and 47000 women infected (UNAIDS 2008) (Figure 1). However, these data do not show the real dynamic of the development of this epidemic, according to temporal changes, geographic distribution, viral diversity and transmission routes.

The global percentage of people living with HIV has stabilized since 2000, probably established by a widely available antiretroviral therapy in low and middle-income countries. However, the overall number of people living with HIV has increased as a result of the ongoing number of new infections every year. Additionally, drug resistance is one of the main challenges faced today in the therapy of HIV infection. Soon after the introduction of the first antiretroviral agents active against

HIV, it became clear that therapeutic regimens using single or dual combinations of antiviral agents were usually unable to yield stable and satisfactory virological and clinical responses. Moreover, the complex outlook results from the high mutation rate of the virus during its replication cycle.

The search for an effective vaccine to control the AIDS pandemic is still continuing long after the discovery and isolation of HIV more than 25 years ago. The complex structure and life cycle of the HIV-1, as well as its high mutation rate, have provided further obstacles to the development of an effective vaccine.

Additionally, HIV escapes from both cellular and humoral immune responses and presently at least 12 known genetic subtypes of HIV-1 which are rapidly diversified to yield intersubtype recombinants: Circulant Recombinant Forms (CRF) and Unique Recombinant Forms (URF), add an even greater challenge to the development of a universal AIDS vaccine.

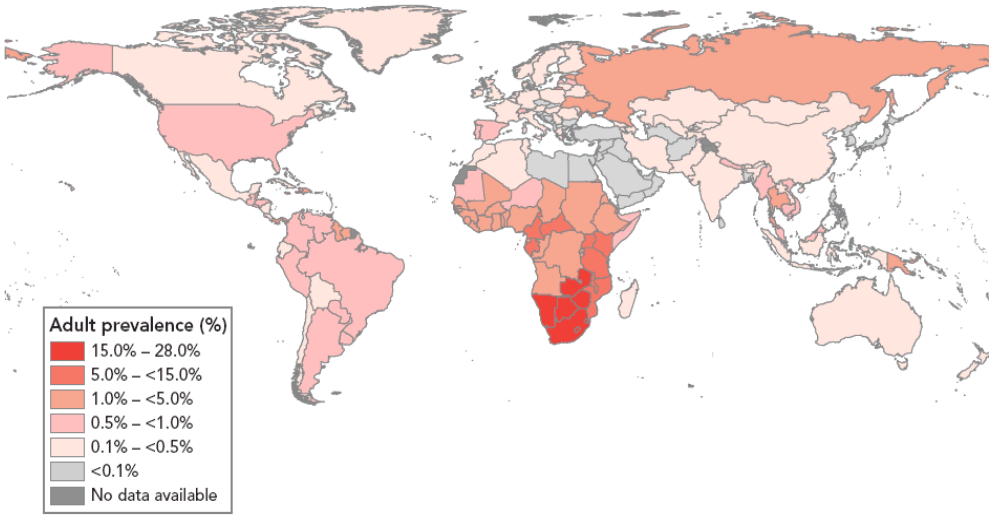


Figure 1. A global view of HIV infection, 2007 (UNAIDS 2008).

Therefore, Genetic therapy may be one such an important therapeutic adjunct. The main objective in gene therapy is the development of efficient, non-toxic gene

carriers that can encapsulate and deliver foreign genetic materials into specific cell types. Notably, the replicative cycle of HIV-1 is vulnerable to interruption at many different points. Consequently, diverse mechanisms have been developed, such as (i) protein-based anti-HIV-1 strategies, including transdominants negative mutants, single-chain antibodies and DNA-based vaccines. (ii) RNA approaches, including antisense, ribozymes, RNA aptamers and decoys, and RNA interference (RNAi).

RNAi is a new method that induces target mRNA degradation with the highest efficiency and specificity achieved until now and holds a promising potential as a novel antiviral treatment. Fully developed, this technology may have the potential to overcome some of the main limitations of gene therapy for AIDS. In addition, it can be used as an alternative treatment regimen against HIV. Inhibition of the expression of key regulatory proteins of the virus using RNAi has demonstrated a dramatic impact on the overall viral replication while allowing proliferation of target host cells. Nevertheless, RNAi-silencing gene is mainly specific of sequence, so it represents an additional challenge due the high mutation rate in HIV, resulting in escape mutants to RNAi activity. Several strategies have been suggested; one of the most promising is to target highly conserved regions that are implicated on precise and essential roles in HIV replication cycle and disease progression. This strategy combined with improvements in efficient and specific delivery into target cells constitutes the primary field in RNAi on HIV research.

1 THEORETICAL CONSIDERATIONS AND STATE OF THE ART

1.1 THE HUMAN IMMUNODEFICIENCY VIRUS

1.1.1 Introduction. The human immunodeficiency virus (HIV) belongs to the Lentivirus genus of the Retrovirus family. There are two types of HIV, type 1 and type 2, but only HIV-1 is worldwide spread. Both pathogens have been transmitted to humans quite recently -20th century. In Africa, non-human primates (NHP) are usually infected with Lentivirus-related species but they do not develop Acquired immunodeficiency Syndrome (AIDS). Chimpanzees infected are resistant to develop AIDS, even though they can have a viral load similar to humans (ten Haaft, Murthy et al. 2001). Therefore, some NHPs especies from Africa are considered as natural reservoirs of Lentivirus (Rutjens, Balla-Jhagjhoorsingh et al. 2003).

HIV has specific features that account for its rapid evolutionary change, such the Reverse Transcriptase lack of proof- reading activity that generates $3.4x10^5$ mutations per bp per replication cycle. HIV genome is approximately 10^4 bp and the viral production rate is 10^{10} virions per day. Thus, in a HIV-infected singular person million of viral variants are produced daily and high recombination frequencies accompanying reverse transcription(Taylor and Hammer 2008). The virus can disseminate within an infected host by two mechanisms: (a) release of cell-free virions and (b) direct passage between infected and uninfected cells. In general, direct cell-cell transfer is more rapid and efficient than cell-free spread because it obviates rate-limiting early steps in the virus life cycle, such as virion

attachment. Moreover, cell-cell passage may help viruses evading elements of the immune response(Johnson and Huber 2002).

Indeed, the HIV infection impairs cells of the immune system, especially CD4+ T cells, because the CD4 differentiation antigen of the T lymphocyte serves as the major cell surface receptor for HIV, which helps to explain the depletion of these cells in AIDS by direct cytopathic effects in activated CD4+ T cells or by syncytium induction. Notably, CD8+ T cells and macrophages become infected via CD4 (Weiss 1993). The transmission routes described are sexual, blood borne and mother-to-child.

1.1.2 HIV history. HIV appears to have been transmitted to humans multiple times from at least two different NHP infected with Simian Immunodeficiency Virus (SIV). Lineages of HIV that were transmitted from chimpanzees (*Pan troglodytes troglodytes*) are known as HIV-1 and are localized in Central Africa, and those transmitted from SIVsm (*Sooty mangabeys*) are known as HIV-2, placed in West Africa. HIV seems to have been transmitted to humans more than once. Regarding HIV-1, the most widespread and devastating epidemic is that caused by the HIV-1 M group, which represents a single lineage with a common ancestor (Hillis 2000). Korber B et al. calculated an estimated date when the last common ancestor of the HIV-1group M came into existence, analyzing the molecular divergence of the *env* gene (encoding gp160). Their molecular clock analysis provided a date of 1931, with a 95% confidence interval of 1915 to 1941 (Korber, Muldoon et al. 2000).

There are three hypotheses trying to explain the origin of the transmission event of HIV-1 group M between chimpanzees (SIVcpz) and human (Hillis 2000). Despite of these studies, direct evidences of virus spread from primates to humans are not fully cleared yet. In particular, it is possible that events as zoonoses were performed also with several complex facts, such adaptations and gain of regulatory viral genes as *vpu*, *rev*, *vif*, *vpr*, *nef* and *tat* until developed a virus able to produce

infection levels enough to be transmissible within a local human population (Heeney, Dalgleish et al. 2006).

1.1.3 The virus biology. The mature virion of HIV-1 is an icosahedral sphere with a diameter of around 100-120 nm. The outer envelope is a lipid bilayer membrane, which is taken from the host cell membrane while the virus is releasing or budding. This bilayer contains host cell proteins and 72 spikes of the viral envelope glycoproteins (gp): gp120 and gp41. Inside the bilayer, the nucleocapsid protein encapsulates two copies of the genomic positive RNA single stranded (ssRNA), and several proteins like: viral Protease (PR), Reverse transcriptase (RT), Integrase (IN), and accessory proteins: Vpu, Vif, Vpr and Nef plus host proteins (Figure 2).

HIV-1 genome is 9.2 kb length, with nine open reading frames which encode 15 proteins including major structural and non-structural proteins common to all replication-competent retroviruses.

From the 5' to 3' ends of the genome are found the gag (for group-specific antigen), pol (for polymerase) and env (for envelope glycoproteins) genes, which are commonly encoded by all retroviruses. The gag gene encodes a polyprotein precursor whose name, Pr55Gag is based on its molecular weight. Pr55Gag is cleaved by the viral protease to the mature Gag proteins matrix (known as MA or p17), capsid (CA or p24), nucleocapsid (NC or p7) and p6. The pol-encoded enzymes are initially synthesized as part of a large polyprotein precursor, Pr160 GapPol. The individual pol-encoded enzymes, PR, RT and IN are cleaved from Pr160 GapPol by the viral PR. The Env glycoproteins are also synthesized as a polyprotein precursor, but are cleaved by a cellular protease instead the viral PR. Gp160 processing results in the generation of the surface (SU) Env glycoprotein gp120 and the transmembrane (TM) glycoprotein gp41 (Frankel and Young 1998).

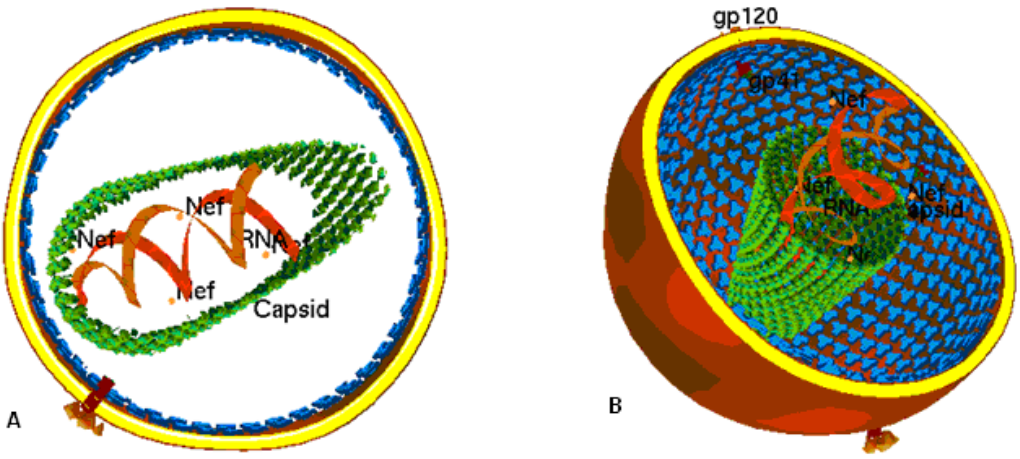


Figure 2. Overview of HIV-1 structure. (a) 2D structure and (b) 3D structure. Structural proteins as gp120 and CA, ssRNA and Nef are being shown as well (Stowell 2002).

In addition to the previous proteins, HIV-1 encodes a number or regulatory and accessory proteins: Tat (Transcriptional activator or p14) is critical for transcription from the HIV-1 Long Terminal Repeat (LTR), Rev (Regulator of viral gene expression or p19) promotes nuclear export of incompletely spliced viral RNAs, Vpu (Viral protein U) promotes CD4 degradation and influences virion release, Vif (viral infectivity factor or p23) overcomes inhibitory effects of host factors, Vpr (viral protein R or p15) promotes G2 cell-cycle arrest, and Nef (Negative factor) promotes down-regulation of surface CD4 and MHC I expression, enhances virion infectivity and alters state of cellular activation (Greene and Peterlin 2002) (Figure 3).

1.1.4 HIV-1 replication cycle. The virus enters by pH independent fusion and uncoats, using the outer surface glycoprotein gp120 for attachment to its main host cell receptor CD4. After binding of gp120 to CD4, conformational changes of the

viral envelope gp120 are initiated, allowing the exposure of the binding site for chemokine receptors (Wyatt and Sodroski 1998). The chemokine receptors used by HIV-1 are primarily CCR5 (Choe, Farzan et al. 1996), found mainly on macrophages and CXCR4 present on many T-tropic primary isolates (Berger, Murphy et al. 1999). HIV-2 and SIV on the other hand, are able to use a broader array of receptors. The HIV-1 is additionally classified according to their coreceptor usage, CCR5-using viruses are known R5 and CXCR4-using viruses as X4. These viruses are non-syncytium-inducing (R5) and syncytium-inducing strains (X4), respectively.

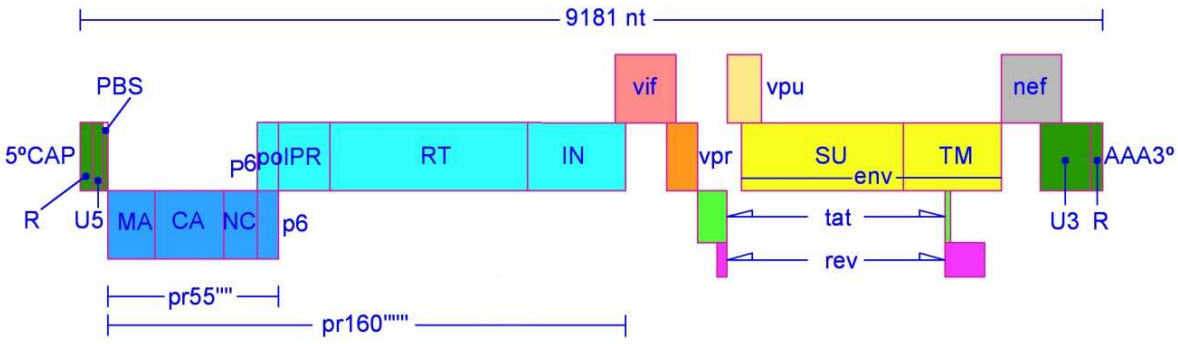


Figure 3. HIV-1 full-genome structure. Structural, regulatory and accessory genes are shown. Modified from Harrich D. Rev Med Virol. 2002;12:31-45.

The interaction of gp120-CD4 complex with the appropriate chemokine receptor triggers additional conformational changes in the envelope glycoprotein complex and results in the insertion of the hydrophobic N-terminal part of Gp41 (the fusion peptide) into the target cell membrane; then a fusion between virus and host cell takes place. This well-known virus-host cell fusion at the cell membrane, has been argued by a recent study showing that HIV-1 uses the endocytic machinery to enter into and fuse with target cells (Miyauchi, Kim et al. 2009).

After viral entry into the cell the RNA genome of the virus is reverse-transcribed by RT into duplex DNA and integrates in the cellular genome by the viral enzyme integrase. The virus can remain latent in this stage for a long period, before it is reactivated and transcribed; replicated, assembled and buds form the cell membrane.

The integrated provirus is flanked by repetitive sequences termed LTR at each 5' and 3' end of the genome, and contains the promoter sequences that drive viral RNA transcription. Once integrated, the transcriptional activity of the provirus is regulated by two virally encoded proteins Tat and Rev as well as other cellular factors inducing host cell activation (Frankel and Young 1998). Early on the viral replication cycle, newly transcribed mRNA is spliced multiple times and the Tat, Rev and Nef proteins are produced (Schwarz, Hutvagner et al. 2003). Once sufficient amount of these regulatory proteins accumulates, synthesis of the unspliced and singly spliced HIV-1 mRNAs encoding the essential structural and enzymatic proteins can proceed. Translation of the Gag and Gag-Pol takes place on ribosomes in the cytoplasm, whereas Env goes through the Endoplasmic reticulum-Golgi-Plasma membrane pathway were it is highly posttranslational glycosylated. The unspliced viral mRNA is translated into the mature viral structural proteins and enzymes. Finally, as all viral components reach the cell membrane; new HIV-1 virions are released acquiring the viral Env proteins as they bud from the host cell membrane. After the budding, the Gag and Gag-Pol polyproteins are cleaved by the viral protease, transforming the immature virus into mature infection virions with a conical core structure (Kaplan, Manchester et al. 1994).

1.2. HIV-Nef: UNRAVELING THE ESSENTIAL MECHANISMS FOR HIV-1 INFECTION BEHIND AN ACCESORY PROTEIN

1.2.1 Introduction. HIV-1, HIV-2, and SIV *nef* was originally designated as 3' open reading frame (ORF) and also as F, ORF B or E, and finally as Nef (negative

factor protein)(Arya and Gallo 1986). In both, HIV and SIV, Nef is encoded by an located at the 3' termini of *env*, and extends into the U3 region of the 3' long terminal repeat (LTR) (Ratner, Fisher et al. 1987). Furthermore, the carboxy-terminal portion of the *nef* ORF overlaps the 3' LTR.
The length of HIV-1Nef is from 8343 to 8963, which correspond to 207 amino acids. In contrast, HIV-2 and SIV are much longer than whole HIV-1 length and Nef length as well. In contrast, HIV-2*nef* encodes a protein of 257 amino acids, and SIV*nef* translates a protein of 263 amino acids.

The regulatory genes *tat* and *rev* and the accessory gene *nef,* are code from a smaller, multiply spliced transcripts present in HIV-1 infected cells (Arya 1987). Robert-Guroff et al. determined that Nef was the most abundant transcript in H9 cells. This finding is independent of the strain of virus and the cell type and is seen with both chronic and short-term infections (Robert-Guroff, Popovic et al. 1990). Moreover, *nef* is the predominant transcript in HIV-infected resting T cells even before integration viral has occurred. Both, *nef* and *tat* expression resulted in an increase in both T cell activation and viral replication, leading to an increase in virion infectivity as well (Wu and Marsh 2001).
Regarding Nef expression throughout the phases of infection in lymphoid cells, it has been shown Nef was produced abundantly 6-9 hours post-infection, similar to Rev and Tat. In contrast, mRNA-encoding gp160 was absent at this period. Also, Nef was abundantly expressed during the productive phase of infection (12-48h post-infection) as full-length mRNA and gp160 was abundant as well (Ranki, Lagerstedt et al. 1994).

The proteins expressed *in vivo* and *in vitro* from *nef* gene include two proteins, one is 25 kDa and the second is 27-kDa. The latter is a full-length translation product of the HIV-1 *nef* gene. Arya et al (Arya and Gallo 1986) and Kaminchik et al., described both forms in cell cultures. The Nef 25-kDa protein is a result from translation of a reading frame initiating at Met-20. It was also shown that Nef 27

kDa undergoes myristyolation resulting in membrane association, as this product shows a long turnover rate in contrast with Nef 25 kDa (Kaminchik, Bashan et al. 1991).

Notably, Nef induces an immune response including monoclonal antibodies and T cells of HIV infected individuals. For instance, studies have showed a prevalence of antibodies against Nef, Env and Gag throughout all clinical phases of HTLV-III infection, being the Nef protein product more immunogenic. It has been also suggested that the essential domains for the immunoreactivity of Nef are located toward the carboxyl half termini (Arya and Gallo 1986).

It has been identified six antigenic epitopes, recognized by sera derived from HIV-1 infected individuals. These epitopes were localized within 8-16 aa, 53-60 aa, 80-100, 115-126 aa, 135-146, 158-170 aa; 180-190 aa and 199-210 aa (Gombert, Blecha et al. 1990). Next, Otake et al. reported other epitopes contained at the N-terminal portion of Nef (aa 18-45), middle of the protein (aa 115-137), and at the C-terminal portion (aa 158-206) (Otake, Fujii et al. 1997).

1.2.2 Nef as a negative factor. Earlier studies on HIV-1, showed *nef* as not essential gene for viral replication and down regulation of transcriptional initiation of HIV-1, hence its name. For instance, transfection of the Jurkat cell line with provirus that contained either a stop codon or deletions within the *nef* showed accelerated replication, in contrast, with the significant delay in the replication if the *env* was mutated (Terwilliger, Sodroski et al. 1986). In fact, Luciw et al, described deletions mutants in the nef region of HTLV-III clone provirus, which were reported to give rise to viruses highly infectious and extremely cytopathic (Luciw, Cheng-Mayer et al. 1987).

On a later study, Ahmad et al showed that *nef* frame shift or premature termination enhanced HIV-1 replication; the repression induced by a normal *nef* was mediated by inhibition of transcription from the HIV-1 LTR (Ahmad and Venkatesan 1988). In the same way, Niederman et al, suggested *nef* may act as a signal transducer,

possibly phosphorylating a cellular protein that may directly or indirectly activate a factor that interacts with the HIV-1 LTR (Niederman, Thielan et al. 1989).

However, further studies did not found concordant results regarding *nef* and its regulatory functions, probably due to different virus strains or cells used in previous experiments. Therefore, some years later the negative conception of *nef* was completely turned out. Shortly, Klester et al, found that animals infected with SIVmac239/*nef*-stop strain, the stop signal quickly reverted to a coding codon; suggesting that *nef* was performing some critical function for the virus life cycle in vivo (Kestler, Ringler et al. 1991). In addition, Zazopolus et al. demonstrated that the *nef* ORF was conserved and *nef* remained functional during passage, displaying a capacity of accelerating virus replication similar to primary *nef* isolates (Zazopoulos and Haseltine 1993). Therefore, the possibility of a positive and important effect of *nef* on the efficient replication and the pathogenic impact on HIV-1 during natural infection has been suggested in several studies.

1.2.3 Post-translational modifications. It has been reported Nef HIV-1 undergoes two post-translational modifications. The first one, the myristoylation: a myristate group anchors to *nef* amino terminal termini, getting a hydrophobic modification. The signal of myristoylation (MGxxx) is localized between 1-6 amino acids and is found to be totally conserved in all *nef* alleles from different subtypes and stages of disease (Shugars, Smith et al. 1993). Unlike Nef from HIV-1, the presence of the myristoyl loop is questionable for HIV-2 and SIV, since in most of HIV-2 strains the Trp5 is replaced by glycine, while in SIV residue 4 and/or 5 is mostly a hydrophobic residue, which might not interact with the myristoyl moiety. Myristoylation induces some conformational changes, which leads to stabilization of two secondary structure elements and form a positively charged cluster including three arginine residues at 17, 21 and 22 amino acids, allowing to direct the Nef protein to the cytosolic membrane (Geyer, Munte et al. 1999).

It has been suggested that Nef protein is capable of associating with the T-cell cytoskeletal matrix and it is facilitated by myristoylation (Niederman, Hastings et al. 1993). The probable area of this interaction could localize at Nef 73-78 aa, and could be implicated in viral transmission from cell to cell and efficient particle release (Fackler, Kienzle et al. 1997). Nevertheless, a specific effect of this interaction has not been disclosed yet.

In a second modification, Nef is phosphorylated by serine protein kinases. The earliest studies implicated this process to the Protein Kinase C (PKC) in threonine at 15 aa (Guy, Kieny et al. 1987). However, Nef from BH10 isolate showed that this residue is not absolutely required for PKC phosphorylation of Nef *in vivo*. In addition, Nef phosphorylation was not completely dependent upon stable association with cytoplasmic membranes (Coates, Cooke et al. 1997). Later, a serin protein kinase in the cyclic adenosine monophosphate (cAMP) signaling pathway namely cPKA, was described as other protein kinase involved on Nef phosphorylation. The specific residue phosphorylated on Nef is Ser9 of HIV NL4-3 isolate and Ser10 of SIVmac239. Particularly, when Nef-Ser^{9} residue was altered, Nef-mediated enhancement of viral replication in resting primary cells was significantly reduced (Li, Wang et al. 2005).

Nef was also found to be associated by its N-terminus with a protein complex, referred to as NAKC (Nef-associated kinase complex), which contains Lck and a serine kinase. The last one phosphorylates Nef as well as Lck *in vitro*. The importance of this association was suggested by mutant studies, where clones altered at N-terminus showed a reduced capacity to infect resting peripheral blood mononuclear cells (PBMC) (Baur, Sass et al. 1997). Similarly, in T cells was found Nef phosphorylated by PKCδ and θ on ser6 residue, and it was relevant for enhance viral transcription and replication (Wolf, Giese et al. 2008).

1.2.4 Nef structure. Previous studies have described two domains in Nef, a smaller N-terminal domain (residues 1-57) and a larger C-terminal domain

(residues 58-203) in HIV NL4-3 isolate. They can be separated in vitro by digestion with proteases, including the HIV protease, which cleaves at a conserved site between residues 57 and 58 (Freund, Kellner et al. 1994).

The full length Nef structure consists of six α-helices (α1- α6) and β-pleated sheet of five antiparallel β-strands (β1- β5)(Grzesiek, Bax et al. 1997). The Nef structure is partitioned in four units, such as a flexible myristoylated membrane anchoring region of variable length (1-56 aa), followed by the PxxP loop (57-80 aa), the core domain (81-206 aa) and a C-terminal flexible loop (148-180 aa).

The architecture of Nef core is constituted mainly by the N-terminal region, which forms an outer layer that consists of a type 2 Proline-rich (PP)-II helix, which contains the P72xxP75 motif, localized between P69xxPxxPxxP78 aa. Through the PxxP motif of this sequence, Nef binds to the SH3 domain of Src kinases. This interaction is among the tightest known SH3-ligand interactions (Lee, Saksela et al. 1996).
Additionally, the N-terminal arm and a 30-aa loop (residues 148-178) are considered as flexible regions, which together comprise 50% of the polypeptide chain. The loop projects out of the core domain and contains three binding motifs, all of which connect Nef with endocytic pathways (Figure 4).

It has been suggested that after translation Nef adopts a “closed conformation” in which its binding sites are hidden and protecting the unstructured parts from proteolytic cleavage. Then, Nef might be translocated to the membrane. The contact with the cellular membrane could trigger a conformational change in Nef, relieving the interaction between the N-terminus and the core, exposing several motifs capable of binding signalling molecules as protein kinases; this form is designed as “signalling conformation”. However, the association of a Nef molecule with the membrane might persist for only a short period. Perhaps a phosphorylation step and/or contact with an appropriate target molecule could lead

to the exposure of the core loop, which change Nef structure to an “open conformation”, enabling bind molecules of the endocytic machinery and mediate internalization of Nef and associated CD4 or MHC-I. The fate of Nef in the last step of the cycle is unclear; although Nef is likely to be degraded, it cannot be excluded that a significant fraction is recycled back to the membrane (Arold and Baur 2001).

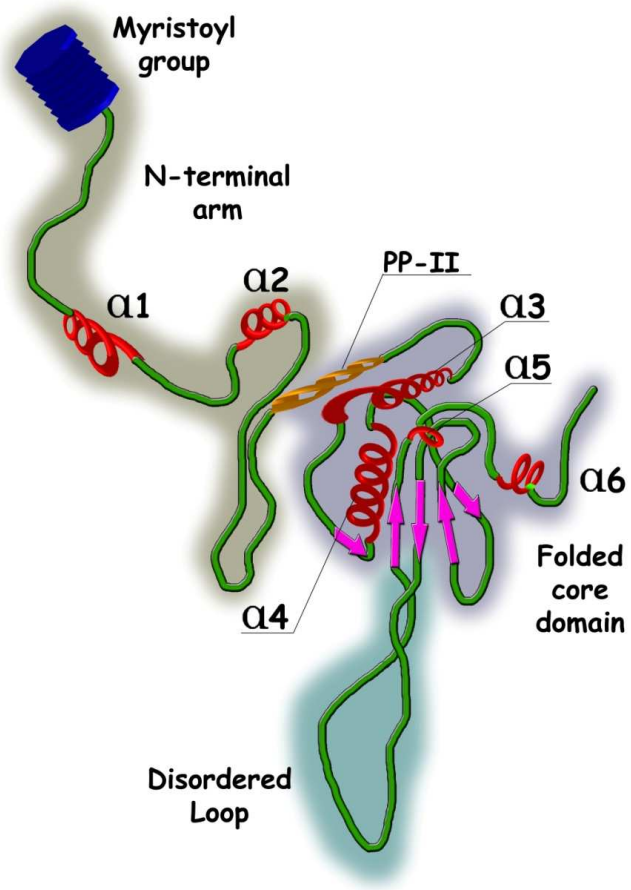


Figure 4. Structure model of the full length, myristoylated HIV-1 Nef. The α helixes and β sheets and important motifs such as Proline-rich PxxPII and Myristoylation signal are displayed. Modified from (Geyer and Peterlin 2001).

Several studies have revealed that Nef forms multimers with itself to form structures including dimers, trimers, and tetramers (Kienzle, Freund et al. 1993).

The connections are principally established by two space groups: R105, D108, I109, L112, Y115, H116 and F121, P122, and D123. These residues are conserved among HIV-1 Nef isolates and form a hydrophobic core (with a hydrophobic “hot spot” central to Y115, F121, and P122) surrounded by charged amino acids (R105, D108, and D123). But, these states of the Nef core domain do not interfere with the recruitment of the SH3 domains and other interactions with molecules implicated in the endocytic pathway. Additionally, Nef may gain affinity for an oligomeric partner, leading to a more efficient interaction.

Oligomerization of Nef may also provide a tool to link proteins that are associated with Nef, i.e., T-Cell Receptor and Src kinases, thus stimulating a functional interaction between these molecules (Arold, Hoh et al. 2000).
Finally, Ye et al showed that Nef oligomers are formed in living cells and localized to the plasma membrane which supports the idea that the Nef dimer is required for Hck kinase activation in vivo and also provides an important proof-of-concept that inhibitors of Nef dimerization may suppress Nef-induced activation of Hck and other kinases important for AIDS pathogenesis (Ye, Choi et al. 2004).

1.2.5 Nucleotide and amino acid conservation. Some researchers have shown that there is a considerable variation in the sequence of the Nef protein. At the aa level, the intra-individual variation ranged from 0.5 to 20.2%, while the inter-individual variation was 0.5 to 22.7%. In addition, at nt level, the intra-individual variation ranged from 1.2 to 11.2%, and the inter-individual variation was 0.3 to 13.2%. This level of both nt and aa sequence diversity is greater than that seen on average in the entire VIH-1 viral genome (Shugars, Smith et al. 1993). In contrast, little evolution of *nef* sequences was noted from the asymptomatic to the disease stage. Among 60 Nef protein sequences studied by Delassus and colleagues, only a few of them were defective: three were truncated by stop codons, one encoded a mutated start codon, two carried large deletions and two were completely defective for replication. Apart from that, some mutations were clustered in the amino and

carboxy termini. But, the region between amino acids 40 and 130 was highly conserved. In addition, the internal sequence variation among Nef proteins was between 1 and 4.5%. Therefore, no predominating defective Nef proteins were found in different phases of HIV-1 infection. (Delassus, Cheynier et al. 1991).

However, Shugars and colleagues described important findings when alignments of different sequences showed several regions and sequence motifs that are highly conserved among HIV-1 *nef* genes obtained directly from infected individuals. First, conserved features present at the N terminus of the Nef sequences included an intact initiator methionine (ATG codon) and a highly conserved myristylation signal (residues 2 to 7). The myristylation signal was followed by a stretch of seven or eight aa that exhibits extensive sequence polymorphism compared with other regions of the Nef sequence. This region may serve as a flexible spacer region and is predicted to lie on the external surface of the folded protein.

Second, the methionine at position 20 has been shown to serve as a site for internal initiation (Shugars, Smith et al. 1993), resulting in a non-myristylated and truncated 25-kDa Nef protein rather than the full-length, myristylated 27-kDa Nef protein(Laurent, Hovanessian et al. 1990).

Third, the Nef sequences also contained a highly conserved sequence including the P72xxP75 motif (Shugars, Smith et al. 1993). The critical role of this motif enhancing viral replication is now well established, because blocking the interaction of Nef with the SH3 domains is possible to interfere with the viral life cycle (Lee, Saksela et al. 1996). Some residues that span this motif are also strictly conserved in Nef sequences and are critical for the interactions with SH3 domain and other components belong to Nef as well. Some are: Arg-77, Val-74, Pro-72 and Pro-75 (Saksela, Cheng et al. 1995).

In addition, another sequence feature can be recognized among the Nef sequences, it is composed mainly of glutamic acid residues (EEEE) and were located at positions 62 to 65 which is highly conserved (Piguet, Gu et al. 1999).

Significantly, the polypurine tract (PPT) was highly conserved. The PPT is one essential element for reverse transcription that is located within *nef* coding sequence. The PPT consists of a purine rich region of 16 nt that lies immediately upstream of the 5' border of the 3' LTR (Pullen, Rattray et al. 1993).

Finally, an unexpected finding was the clustering of premature stop codons at position 124 (normally encoding tryptophan) in six sequences derived from 4 of the 12 individuals, suggesting that truncated Nef proteins may be generated in vivo and may display altered properties compared with the full-length Nef protein (Shugars, Smith et al. 1993).

Recently, Geyer et al. have reported that single aa deletions are found predominantly at three locations: From position 8 to 11 (5%), 49 to 51 (7%) and 155 to 164 (3%). These locations correlate with lower sequence conservation and structurally less defined loop regions. Also, the most homogenously conserved part of Nef ranges from V66 to V148 which includes the polyproline helix and the β-strands (Geyer and Peterlin 2001) (Figure 5). On the other hand, significant variability in at least one of the residue was described on E154/E155 (diacidic), R105/R106 (diarginine), and E62/E63/E64/E65 (polyacidic) (O'Neill, Kuo et al. 2006).

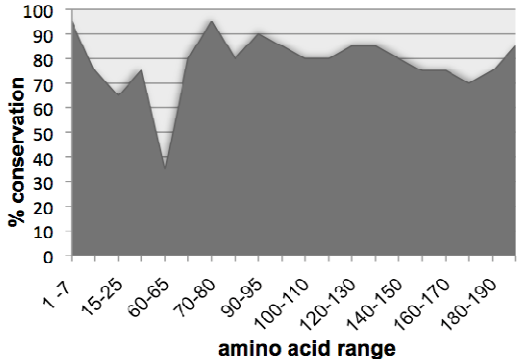


Figure 5. Sequence conservation percentage in full-length Nef sequence. Regions highly conserved are displayed. Adapted from (Geyer and Peterlin 2001).

In conclusion, so far Nef has been extensively analyzed by mutational studies in several efforts to elucidate its determinants to achieve all its functions. However, there are not complete and supported studies that integrate all conserved aa and sequences within the complicated structure of Nef with specific interactions to a given residue during the infection cycle.

1.2.6 Subcellular localization of Nef. One of the first studies about localization of Nef in the cell was developed by Franchini et al. The authors detected both Nef 25 and 27 kDa in both cytosol and membrane fractions, but not in the nuclear or organelle fractions (Franchini, Robert-Guroff et al. 1986). In contrast, Kaminchik et al, differenced Nef 27 kDa protein expressed both in the cytosol and membrane fractions, whereas the 25-kDa was present exclusively in the ctyosol due to the lack of myristoylation (Kaminchik, Bashan et al. 1991). Regarding the Nef expression in the different stages of the infection cycle, the subcellular localization of Nef changed according to those phases. For instance, up until 24 h post-infection Nef was expressed almost exclusively in the cytoplasm with a characteristic polar pattern, indicating localization in the Golgi complex and endoplasmic reticulum (Mangasarian, Foti et al. 1997). Also, Fackler et al. analyzed the distribution of Nef G2A mutant, which was distributed more diffusely in infected cells and was also found within the nucleus presumably due to the loss of myristoylation. In contrast, Nef wild type was found more predominantly enriched in the perinuclear areas of infected cells, with some cells showing marked staining of the plasma membrane and intracellular vesicles (Fackler, Moris et al. 2006).

1.2.7 Nef and its functions

1.2.7.1 CD4 down-regulation. The earliest evidence about Nef and its functions on enhancing HIV-1 replication showed an essential negative regulation on CD4 receptor on host cells. These studies established that the cytoplasmic domain of

CD4 is required for its down-regulation by Nef. It was confirmed by a deletion of the cytoplasmic domain of CD4 that resulted in lack of down-regulation by Nef (Garcia, Alfano et al. 1993).

About the essential region in Nef for CD4 degradation, some aa have been proposed, for example: W57, L58, E59, G95, G96, L97, R106 and L110 (Grzesiek, Stahl et al. 1996). In addition, a highly conserved dileucine motif (EXXXLL) located at residues 164-165 were vital to down-regulate CD4 by HIV-1 Nef (Bresnahan, Yonemoto et al. 1998). Recently, it was found a new sequence highly conserved among all strains of HIV-1 and SIV important to CD4 down-regulation: a diacidic pair DD (residues 174 and 175). This sequence was absolutely required for directing binding of Nef to adaptor protein type 2 (AP-2) (Lindwasser, Smith et al. 2008). Also, Jin et al. demonstrated that dileucine sorting motif-based endocytosis of CD4 and HIV/SIV Nef is AP-2 dependent and is being assisted by other components of the clathrin endocytic machinery (Jin, Cai et al. 2005). Additionally, Nef has been localized to plasma membrane clathrin-coated pits that contain AP-2 (Greenberg, Bronson et al. 1997).

The CD4 down-regulation by HIV-1 and SIV Nef involves internalization and intracellular retention mechanisms. First studies showed that Nef promoted CD4 endocytosis, which was followed by degradation of CD4 in lysosomes. This phenomenon was myristoylation-dependent and was manifested at an early stage during HIV infection (Aiken, Konner et al. 1994). Next, Rose et al showed intracellular retention of CD4 pre-synthesized endogenously in both lymphocytes and epithelial cells (Rose, Janvier et al. 2005). Its retention could include nascent CD4 encounters which bind to Nef in some vesicular compartment, consequently to lend the formation of a CD4-Nef-AP2 complex, which is retained and eventually redirected to a degradation pathway (Giolo, Neri et al. 2007).

Notably, Nef mutants at L168/169A, ED178-179AA were unable to interact with the endocytic machinery. Also, Δ59-61 and Δ12-39 variant failed to down-modulate the CD4 receptor. These results revealed that all Nef variants without CD4 down-

regulation activity, failed to enhance HIV-1 replication in Pheripheral Blood Lymphocytes (PBL). Perhaps, reflecting the role of Nef-mediated CD4 down-regulation on HIV-1 replication, infectivity, particle release and super infection of already productively infected cells (Glushakova, Munch et al. 2001; Fackler, Moris et al. 2006). Also, Nef-dependent down-regulation of CD4 in virus-producing cells was shown to favor efficient HIV-1 envelope glycoprotein incorporation into virions by preventing the formation of CD4/gp120 complexes at the cell surface (Arganaraz, Schindler et al. 2003).

1.2.7.2 Class I MHC down-regulation. The second Nef essential function found was the interference with Class I MHC expression at the cell surface, protecting infected primary cells against killing by cytotoxic T lymphocytes and allowing Nef-expressing cells survive longer *in vivo* to produce more infectious viral particles (Collins and Baltimore 1999).

Remarkably, Nef requires an intact SH3 domain-binding surface, promoting the accumulation of class I MHC complexes, internalized from the plasma membrane in the trans-Golgi (Greenberg, Iafrate et al. 1998). Additionally, a cluster of acidic aa residues conserved in HIV-1 Nef at positions 62-65 composed by four glutamic acid (EEEE) is necessary (Shugars, Smith et al. 1993). By mutational analysis a methionine at position 20 (M20) was critical on class I MHC down-regulation (Akari, Arold et al. 2000). Additionally, the removal of Nef myristoylation site prevents both CD4 and MHC-I negative regulation (Peng and Robert-Guroff 2001). In particular, G2A, E4A4 and RRLL variants failed to down-regulate MHC-I, and only the Δ12–39 variant displayed an intermediate phenotype (Fackler, Moris et al. 2006).

The determinants required in MHC for Nef-induced modulation were located in the cytoplasmic domain. It is characterized by the sequence Y320SQA323, and is recognized as a Tyrosine-based sorting signal interacting with the medium chains (μ1 and μ2) of clathrin-associated AP complexes. In the presence of Nef, MHC

HLA-A and –B molecules are accumulated in a perinuclear region probably inducing the accumulation of MHC I in clathrin-coated vesicles (Le Gall, Erdtmann et al. 1998). Interestingly, HLA-C, HLA-E is not down-regulated by HIV. HLA-C and HLA-E left on the surface of HIV-infected cells protect those cells from NK lysis (Cohen, Gandhi et al. 1999).

First evidence about MHC-I down-regulation mechanisms, suggested that Nef disrupts MHC-I by direct binding to the MHC-I cytoplasmic tail and promoting its association with other cellular proteins such as phosphofurin acidic cluster sorting protein 1 (PACS-1) (Cohen, Gandhi et al. 1999). Also, Nef disrupts MHC-I trafficking after it reaches to the Trans-Golgi network (TGN) to prevent MHC-I cell surface expression and to promote its degradation in lysosomes. This activity of Nef required the expression of AP-1A, but not AP-3 (Roeth, Williams et al. 2004). Hung et al. delimited a likely signaling pathway regarding this Nef effect, it includes Nef-Src Family tyrosine kinases (SFKs)-ZAP-70/Syk-PI3K complex that stimulates the Adenosine diphosphate-ribosylation factor-6 (ARF6)-controlled endocytosis of cell-surface MHC-I (Hung, Thomas et al. 2007). A recent study added PACS-2 and PACS-1 to this pathway. First, PACS-2 is required for localization of HIV-1 Nef to the paranuclear region, being dependent of the acidic cluster $EEEE^{65}$-Nef, which enables Nef to assemble the SFK-cascade, $PXXP^{75}$-dependent, triggering down-regulation of cell-surface MHC-I. Second, PACS-1 mediates recycling to the cell surface (Atkins, Thomas et al. 2008). Additionally, new evidence suggested that Nef uses β-coatomer protein (β-COP) to promote trafficking to degradative compartments in both main Nef functions. Thus, proposing that AP-1 and AP 2 deliver MHC-I and CD4 respectively to endosomal compartments where β-COP displaces AP-1 and AP-2 to target MHC-I and CD4 for lysosomal degradation. The principal domain on Nef to interact efficiently with β-COP is localized at D123 residue (Schaefer, Wonderlich et al. 2008).

Notably, HIV-1 strains isolated from rapid progressors showed a prominent CD4 inhibition but it was less effective on MHC class I down-regulation. Alternatively,

the HIV-1 strain from slow progressors showed the highest down-regulation of MHC Class I, whereas CD4 inhibition was the lowest effect (Yoon, Jeong et al. 2001).

1.2.7.3 Nef and kinase interactions in signaling pathways. Like CD4 and MHC class I down regulation, Nef-full length has shown a similar effect on Interleukin-2 receptor (IL-2R) in CD4+ T cell lines (Greenway, McPhee et al. 1994). Nef expressed in PBMC also inhibited IL-2 and it was found to interact with several cellular proteins like p56lck, p53, MAPK/ERK 1, which are implicated in cell activation and cell cycle. In addition, decreasing T cell activation Nef might inhibit virus-induced apoptosis (Greenway, Azad et al. 1995).

The principal protein interaction involving Nef is the binding to Hck and Lyn kinases, mediated by the PxxP motif (Saksela, Cheng et al. 1995). Nef interaction with Hck promotes Hck autophosphorylation activating its tirosyne kinase activity at T cell activation pathway (Moarefi, LaFevre-Bernt et al. 1997). Also, Nef can promote virus replication and dissemination by Hck-Stat3 pathway in a monocyte/macrophage precursor cell line (Briggs, Scholtz et al. 2001). The subsequent signaling requires oligomerization of Nef at plasma membrane and at least two Hck monomers for autophosphorylation (Ye, Choi et al. 2004). Other residues have been reported to be important on Hck interaction: Tyr120, Phe90 and Trp113 (Choi and Smithgall 2004).

In T cells, the initial signaling event following ligation of the antigen receptor is the activation of Src family tyrosine kinases and the subsequent phosphorylation of ITAM motifs in the TCRζ cytoplasmic tail, resulting in the recruitment of ZAP-70 and upregulating 15 transcription factors such NFATc, c-fos, JUND, IRF-1 and c-myb that transactivate the LTR (Simmons, Aluvihare et al. 2001). Nef affects T cell activation through the increase in the association of both TCRζ and Lck with rafts. This process is dependent on Nef myristoylation (Djordjevic, Schibeci et al. 2004).

Rafts-Nef association enables to recruit Nef into the Immunological synapse (IS) after TCR engagement (Fenard, Yonemoto et al. 2005). Alternatively, Nef modified endosomal traffic of Lck and TCR, so impairing the formation of the IS, probably resulting in the inhibition of apoptosis in the infected cells and the establishment of latently infected lymphocytes (Thoulouze, Sol-Foulon et al. 2006).

Modulating cellular activation could not only lead to increased virus replication and infectivity of viral particles produced in the infected cell, but could also promote spreading of HIV to neighboring cells through their paracrine stimulation. Additionally, altering the physiology and antiviral responses in the infected cells, Nef could also modulate apoptosis in these cells, as well as in cells involved in antiviral defense, or affect the balance of cytokine networks of the immune system in ways that may promote progression of the infection (Saksela 1997).

1.2.7.4. Additional functions. The discoveries about Nef and its functions have led to discover new key roles on viral infectivity and HIV-1 infection cycle in host cells, especially the effects on reverse transcription.

Nef protein is incorporated into virions, approximately 5-10 molecules per virion, with PR, Gag and Pol proteins (Welker, Kottler et al. 1996). In particular, the signal for virion incorporation possibly include residues from 7 to 22, which consists of covalently attached myristic acid and a cluster of positive charges in the N terminal region of the protein (Welker, Harris et al. 1998). Nevertheless, it has been recently reported that the presence of Nef in viral particles does not suffice to increase viral infectivity (Laguette, Benichou et al. 2009).

Moreover, processing events are performed in the virions by the viral protease yielding a Nef fragment of 20 kDa. The sequence target to protease is ACAWLEAQ, which is highly conserved among HIV-1 isolates. The mutations on this region highly influence the decrease of infectivity (Pandori, Craig et al. 1998).

However, Chen Y et al, showed that the cleavage of Nef is not essential for its ability to enhance virion infectivity (Chen, Trono et al. 1998).

Miller and colleagues in 1994 demonstrated that HIV-1 *nef* significantly augments the in vitro replication of HIV clones on primary T lymphocyte and macrophages. However, this effects are only visible when the cells are activated following infection but not when previously activated cells were used (Miller, Warmerdam et al. 1994). Similarly, Münch et al. showed that group M, N and O Nef also enhance virion infectivity, viral replication and cytopathicity in PBMCs and lymphoid tissue ex vivo (Munch, Rajan et al. 2007).

In addition, the 3' end of the *nef* sequence overlaps the modulatory U3 region, which is part of the LTR and contains the important elements as the basal promoter, core enhancer, a long modulatory region and the polypurine tract (PPT) as well (Gaynor 1992). It has been shown that HIV-1 lacking the modulatory U3 region and the *nef*-LTR overlapped region can replicate efficiently and cause CD4+ T cell depletion in human T cells and ex vivo-infected lymphoid tissue. But, the mutant *nef* will most likely not induce disease as consistent as wild type HIV-1 strains (Munch, Rajan et al. 2005).

It has been demonstrated that the reverse transcription plus-strand synthesis initiates from two well-defined sites within the HIV-1 genome: the 3' and central PPT. The precise plus-strand initiation from the 3' PPT is a critical stage in reverse transcription because the truncated 5' LTR would delete several critical transcriptional control elements.(Rausch and Le Grice 2004). The plus-strand DNA primer is provided by a 15- nt purine-rich viral RNA: 5´-AAAAGAAAAGGGGGG-3´, and is generated from viral RNA by the RNase H activity of RT, being highly conserved in most retroviruses (Pullen, Rattray et al. 1993). McWilliams et al demonstrated that generating multiple mutations in the first half of the PPT could alter the specificity of RNase H cleavage and affecting significantly virus titer in a single cycle of retroviral replication (McWilliams, Julias et al. 2003). Equally,

changing four G's at the 3' end of the PPT to four C's resulted in a dramatic loss in priming activity (Powell and Levin 1996).

Finally, the functions described here have allowed establishing a general picture of how Nef is becoming an important protein on HIV-1 infection cycle, especially altering cellular trafficking, signal transduction, infectivity and pathogenesis. Although, the final picture is not ready yet, important new findings add lights to outline Nef role. In fact, all previous and future data may provide a full understanding on Nef and permit to design new therapies focused on block HIV infection cycle.

1.2.8. Nef and Long Term non-progressors. Several viral and host factors and their interactions, play key roles in the pathogenic process of HIV-1 infection, making it complex and variable, resulting on differential viral virulence and disease course. Most people after seroconversion develop AIDS-related symptoms within a period of approximately 10 years. However, several studies have described a small population of infected individuals, namely Long-term survivors or long-term nonprogressors (LTNP), who remain clinically healthy and immunologically normal (Huang, Zhang et al. 1995). Individuals in this group maintain CD4+ positive lymphocyte counts greater than 500 cells/ml without receiving therapy.

Some defects in the viral genome, including some mutations at accessory proteins as *vif*, *vpr*, *vpu* and *nef* genes have been reported (Mariani, Kirchhoff et al. 1996). The first study linking *nef* and its role on progress of HIV-1 infection to AIDS, described a cohort of seven individuals from Australia infected with a common *nef*-defective strain of the virus after being transfused with blood products from a common donor. All members of the cohort remained stable for 14 years, and their amounts of HIV-1 DNA were very low. There was a deletion of ~160 to 430 bp from the *nef*-LTR region, even though all cohort sequences studied have an intact PPT, Sp1 basal promoter region, TATA box and TAR sequences; features which are all crucial for virus replication (Deacon, Tsykin et al. 1995). In addition, *nef* alleles

derived from a LTNP carried unusual deletions of 36-bp and a duplication of 33-bp close to the 5' end. This duplication restored the ability of Nef to increase virion infectivity and the ability to down modulate MHC-I, but CD4 down-regulation function was inactive (Carl, Daniels et al. 2000). However, after more than 16 years of infection, three of the living Australian cohort members including the donor have declining CD4 cell counts and viral loads have raised from 1,000 to 10,000 copies/ml (Learmont, Geczy et al. 1999).

Especially features of Nef sequences associated with different stages of HIV disease showed that most previously defined conserved domains in Nef were conserved in Non progressors (NP), slow progressor (SP) and rapid progressors (RP), especially the $PxxP_3$ motif and the ExxxLL motif. However, changes in NP and SP were observed in the N-terminal myristoylation, also additional G or K residue in the middle of the acidic charged region (71-75 aa) and some variations on aa that could be phosphorylated (Kirchhoff, Easterbrook et al. 1999).

Remarkably, *nef* has been strongly associated with pathogenesis in vivo. For instance, infection of rhesus macaques with viruses derived from infectious molecular clones of SIV lacking *nef* showed low viral load, normal circulating CD4+ T-cell counts, and no signs of disease progression (Kestler, Ringler et al. 1991). Furthermore, these animals were "vaccinated" in a challenge with wild-type (WT) virus. Rhesus monkeys vaccinated with SIVmac239/nef-deletion appear to have resisted challenge by wild-type pathogenic SIV. And genetic analysis of the recovered virus and viral DNA present in their PBMCs revealed no evidence of wild-type *nef* challenge virus (Daniel, Kirchhoff et al. 1992).
Finally, monkeys immunized with the attenuated SHIVs (chimeric simian and human immunodeficiency virus) were able to develop protective immunity from the disease progression by a heterologous pathogenic virus infection early after vaccination, without decreasing on CD4+ T cells (Enose, Kita et al. 2004).

1.3. RNA MEDIATED GENE SILENCING

1.3.1 Introduction. Post-transcriptional gene silencing (PTGS) occurs in plants and fungi transformed with foreign or endogenous DNA and results in the reduced accumulation of RNA molecules with sequence similarity to the introduced nucleic acid. Double-stranded RNA (dsRNA) induces a similar effect in nematodes, insects and protozoa. PTGS can be suppressed by several virus-encoded proteins and is closely related to RNA-mediated virus resistance and cross-protection in plants. PTGS could also represent a defense system against transposable elements and may function in plant development. PTGS induced by transgenes can also occur when a transgene does not have homology to an endogenous gene (Hamilton and Baulcombe 1999).

The history of RNAi has unfolded rapidly since 1997 with a series of discoveries from plants, fungi and animals. However, the discussion about co-suppression of an endogenous gene with a transgene started in 1990, when an approach designed to enhance floral coloration by over-expression of flavonoid genes resulted in a reduction in floral pigmentation, indicating that this inhibition was possibly caused by a specific reduction in mRNA steady-state level of the gene of which extra copies had been added to the genome (van der Krol, Mur et al. 1990).
Further, Fire et al. suggested a similar interference mechanism in the expression of two genes encoding myofilament proteins present in *C. elegans* (Fire, Albertson et al. 1991). This disruption was suggested at a late step in gene expression, such as transport into the cytoplasm or translation. In a subsequent study, Fire et al. described the requirements and the central features of this mechanism:
(i) The sense-antisense mixture produced highly effective interference with endogenous gene activity compared with single strand RNAs.
(ii) This inhibitory effect was only observed when dsRNAs sequences matched a region within the target gene.

(iii) dsRNA-mediated interference showed a surprising ability to cross cellular boundaries.
(iv) The robust interference was found in the progeny of both gonad arms (Fire, Xu et al. 1998).
Now, it appears that RNAi-mediated gene silencing is an important component of the eukaryotic immune response to viral infection that has been highly conserved in evolution. Short dsRNA molecules also appear to play an important role in gene regulation in plants, animals and fungi. Moreover, the RNAi technology has been employed in different fields of biomedical research: validation of disease models in vitro, drug activities through the removal of suspected targets, identification of new drug candidates in genome-wide, functional genomic screens and in gene therapy as therapeutic molecules in the clinic (Vanhecke and Janitz 2005).

1.3.2 RNAi in mammals. The principal function of the RNAi pathway during eukaryotic evolution included a cell-based defense against viral and genetic parasites. dsRNA viruses and mobile genetic elements have the potential to form dsRNA structures and can be subjected to RNAi-dependent gene silencing in *C. elegans*, plants, Drosophila and mammals (Zamore and Haley 2005).
Studies conducted on these organisms have demonstrated that 21-25 nucleotide (nt) RNA fragments, referred to as short interfering RNAs (siRNAs), are essential sequence-specific mediators of RNAi, and those siRNAs are generated from long dsRNAs by digestion with an RNase III-like nuclease, Dicer (Hohjoh 2002). Interestingly, among these distantly related organisms, the different mechanistic aspects of the RNAi pathway have diverged, making the use of RNAi for gene silencing species-dependent.

The core pathway appears to be well conserved: dsRNA enters the cell cytoplasm either through the transcription of endogenous microRNAs (miRNAs), viral delivery or complex formation with a lipid carrier and endocytosis, where it encounters the Dicer enzyme complex. This complex has two RNase III motifs, an RNA helicase

domain and a dsRNA-binding domain (dsRBD). Dicer cuts the dsRNA into 19-21 base pair (bp) fragments typically with a 5' phosphate group and a two-base overhang at the 3' end (Bernstein, Caudy et al. 2001). The complex generating the siRNAs from short dsRNAs primarily recognizes the 3´termini of the duplex. The Dicer's domain responsible for siRNA processing are PAZ and two RNAIII, which act as a molecular ruler to precisely measure out ~21-nt siRNAs for cleavage (Figure 6) (Zhang, Kolb et al. 2004).

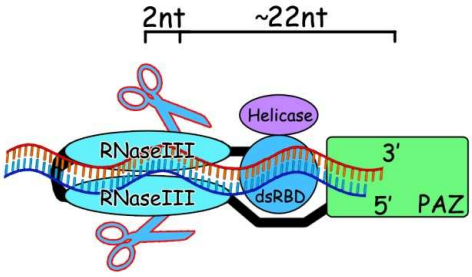


Figure 6. Model for Dicer catalysis. The PAZ domain binds the 2nt 3' overhang of a dsRNA terminus. The RNAse III-Dicer domains cut a dsRNA molecule generating siRNAs of ~22nt including a two-base overhang at the 3' end. Adapted from (Zhang, Kolb et al. 2004; Hammond 2005).

The siRNAs then serve as sequence-specific guides to an effector's complex called the RNA-induced silencing complex (RISC) that carries out the destruction of homologous mRNAs (Elbashir, Martinez et al. 2001). In 2005, Chendrimada and colleagues, found that Dicer interacts with a dsRNA binding protein called TRBP (TAR RNA binding Protein), connecting Dicer into the RISC complex through the interaction with the Argonaute 2 (Ago2) in miRNA-silencing pathway (Chendrimada, Gregory et al. 2005). However, the function of TRBP was changed, as it was demonstrated that TRBP is required for the assembly of RNA silencing complexes mediated by siRNAs and endogenous miRNAs (Haase, Jaskiewicz et al. 2005). Further studies added one more dsRNA binding protein to Dicer partners, PACT whose function was suggested at the RISC assembly (Lee, Hur et

al. 2006). Recently, it has been shown that Dicer directly interacts with TRBP and PACT and they facilitate the production of siRNAs (Kok, Ng et al. 2007).

The core of RISC complex comprises a protein namely Ago2, which possesses the slicer activity of RNAi (Liu, Carmell et al. 2004). Ago2 is composed of three domains: the PAZ domain at the N-terminus, the “middle” domain and the PIWI domain at the C-terminus. The siRNA is unwound in a strand-specific manner during RISC assembly. This single-stranded siRNA locates mRNA targets by Watson-Crick base pairing. It has been demonstrated that the cleavage of the target strand by the PIWI domain, is about 11-nt away from the 5’ end of siRNA antisense molecule (Song, Smith et al. 2004). Remarkably, it has been found that each RISC contains only one of the two strands of the siRNA duplex (Martinez, Patkaniowska et al. 2002). Both siRNA strands can be competent to direct RNAi (Elbashir, Martinez et al. 2001). However, designing siRNAs that are fully asymmetric, only one of the siRNA strands will form RISC in vitro. It means that the anti-sense strand of an siRNA will direct the cleavage of a corresponding sense RNA target while the sense-siRNA is degraded, avoiding undesirable effects (Schwarz, Hutvagner et al. 2003). RISC has dual functions which direct the target mRNA either for cleavage or for translational repression, depending on the base-pairing features between the small RNA and the target mRNA (Hutvagner and Zamore 2002). Therefore, mRNA cleavage or translational repression based on its complementarities: if it is perfect, induces degradation, whereas several mismatches lead to translation arrest (Zeng, Yi et al. 2003) (Figure 7). The degradation of mRNA by RNAi occurs in the cytoplasm. The more specific location has been suggested to be the cytoplasmic processing bodies (P-bodies). Nevertheless, in a recent work this function for P-bodies has been discussed, since P-bodies arise as a consequence of the silencing pathway (Eulalio, Behm-Ansmant et al. 2007).

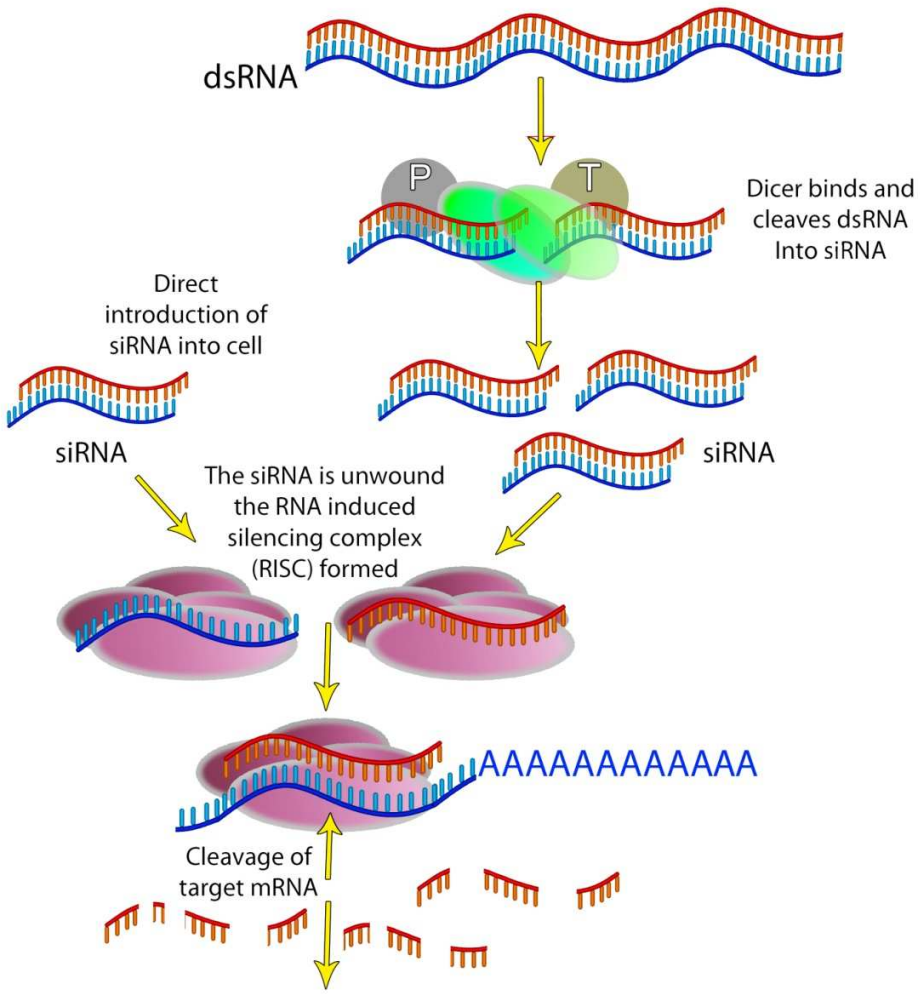


Figure 7. General view of siRNA-silencing pathway. The cleavage of target mRNA is caused by dsRNAs and siRNAs that trigger the interference silencing in vitro. Adapted from (Azorsa 2003; Kok, Ng et al. 2007).

1.3.3 microRNAS in mammals. The miRNA is a form of small, single-stranded RNA (ssRNA), 18- to 25-nt long. It is transcribed from DNA and instead of being translated into protein, regulates the functions of other genes in protein synthesis. Ambros et al. were the first researchers to describe members of these non-coding RNA group: *Lin-4* and *let-7*. These miRNAs are produced directly from protein-coding sequences and have precise antisense complementarity to the 3'

untranslated regions (UTR). In addition, they were identified as key players in the control developmental timing in *C. elegans* (Lee, Feinbaum et al. 1993; Ambros, Lee et al. 2003).

New findings have allowed enhancing the knowledge of miRNA roles in plants and mammals. Important functions in the regulation of apoptosis and cell proliferation in fruit flies, neuronal asymmetry in *C. elegans*, hematopoietic differentiation and abnormal miRNA expression in tumor cells in humans have been described (Mendell 2005).

The miRNAs are processed from the intergenic regions and introns of protein-coding host genes transcribed by Pol II as pri-miRNAs (Lee, Kim et al. 2004). The pri-miRNAs can be quite long, more than 1kb and loops interrupt the structure. In the nucleus, pri-miRNAs are processed by an endonuclease RNAse III namely Drosha, which cleaves both strands of the steam at sites near the base of the primary steam loop (Lee, Jeon et al. 2002). Drosha is contained within a large (500-650 KDa) nuclear complex dubbed the “microprocessor” (Denli, Tops et al. 2004), where it interacts with a cofactor, DGCR8/Pasha, which may initiate binding to the pri-miRNA and is essential for its activity (Han, Lee et al. 2004). The cleavage of the pri-miRNA is determined by the ssRNA-dsRNA junction at the base of the miRNA hairpin, where Drosha cuts approximately 11bp from the base (Han, Lee et al. 2006), liberating a ~60-70 nt stem loop intermediate, known as the miRNA precursor, or the pre-miRNA (Zeng and Cullen 2003), which has a 5’ phosphate and ~2nt 3’ overhang (Lee, Ahn et al. 2003). Then, the pre-miRNA is actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Exportin-5 (Lund, Guttinger et al. 2004). The nuclear cut by Drosha defines one end of the mature miRNA. The other end is processed in the cytoplasm by the enzyme Dicer (Bernstein, Caudy et al. 2001). Dicer first recognizes the double-stranded portion of the pre-miRNA, perhaps with particular affinity for a 5’ phosphate and 3’ overhang at the base of the stem loop. This cleavage by Dicer left the 5’ phosphate and ~2 nt 3’ overhang characteristic of an

RNase III and producing an siRNA-like imperfect duplex that comprises the mature miRNA and similar-sized fragment derived from the opposing arm of the pre-miRNA. Following cleavage by Dicer, the effectors pathway is equally to siRNAs. Likewise, miRNAs can direct the RISC to downregulate gene expression by either of two posttranscriptional mechanisms: mRNA cleavage or translational repression (Zeng, Yi et al. 2003).

1.3.4 Triggers of RNAi and delivery systems in mammals. Soon after the discovery of RNA-silencing mechanisms in *C. elegans*, Thuschl and colleagues demonstrated that RNAi can be triggered in several cell lines through the introduction of synthetic siRNAs (ssiRNAs), mimicking the products of the Dicer enzyme (Elbashir, Harborth et al. 2001). Later on, it was shown that 29-nt synthetic short-hairpins RNAs (shRNAs) with the 3' overhang was converted predominantly into a specific and unique small RNA product of 22-nt cleavages by Dicer (Siolas, Lerner et al. 2005). However, the principal disadvantage of using these ssiRNAs is its transient suppression effect. In particular, this weakness was solved by the endogenous expression of shRNAs, which suppress gene expression in mammalian cells stably. These RNA duplexes harboring different siRNA motifs separated by ssRNA loops, with a structure of N19 (sense strand)-loop-N19 (antisense strand), can be provided exogenously or can be synthesized in vivo from RNA polymerase III promoters (Leirdal and Sioud 2002; Paddison, Caudy et al. 2002). Brummelkamp et al. designed the first approach of a mammalian expression vector that directs the synthesis of siRNA-like transcripts, containing two 3' overhanging T or U nt. The vector, namely pSUPER (suppression of endogenous RNA) uses the polymerase-III H1-RNA gene promoter. The small RNA produced from this vector lacks the polyadenosine tail, with a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5). The knockdown mediated by pSUPER was maintained over long periods without any toxicity in the cells (Brummelkamp, Bernards et al. 2002). The efficiency in gene silencing mediated by shRNAs was improved by the design of a

retroviral vector, Long Terminal Repeat (LTR)-modified, the pRETRO-SUPER (retroviral suppression of endogenous RNA), which permits to integrate expression cassettes in the genomes of human cells, inducing persistent loss-of function features (Brummelkamp, Bernards et al. 2002). Vectors expressing shRNAs have been greatly used, driven by different promoters such as the human U6 small nuclear RNA (snRNA) promoter (Paul, Good et al. 2002) or mouse U6 promoter (Yu, DeRuiter et al. 2002). Some new approaches have been evaluated in order to improve shRNAs delivery among them:

(i) The construction of multiple shRNA expression vector driven by four different Pol III promoters: Human and mouse U6 Promoter, Human H1 Promoter and 7SK promoter. The expression of multiple shRNA simultaneously effectively improves the efficiency of knockdown over a single shRNA construct (Gou, Weng et al. 2007).

(ii) The substitution of the stem sequences of the mir-30 precursor with unrelated base-paired sequences driven by Pol II promoters, have been demonstrated to be useful for the expression of siRNAs-like sequences in human cells (Zeng, Wagner et al. 2002).

(iii) The construction of vectors producing a synthetic miRNA perfectly complementary to a target mRNA by substituting the stem sequences of the mir-155 precursor and optimizing it to express multiple expression cassettes from a single transcript. It has showed enhancing target inhibition (Chung, Hart et al. 2006).

(iv) Lentiviral vectors constitute probably the best choice available for delivering and stably expressing shRNAs or siRNAs targeting single or multiple gene products in several cell types. Moreover, systems with additional features such as tetracycline (Tet)-inducible expression of miR-shRNAs (Shin, Wall et al. 2006) or lentiviral vector system induced by CRE recombinase have demonstrated considerable downregulation of several target genes (Heinonen, Mohamed et al. 2005).

In brief, although shRNAs delivery represented a problem at first, it has been greatly improved with new advances that properly use infective properties of virus to effectively deliver shRNAs both *in vitro* and *in vivo* into specific cells. Some drawbacks still remain, but new approaches are continuously being developed.

1.3.5 siRNA design. While siRNA expressing vectors have been developed, several attempts have been made to discover the requirements regarding how to design siRNA duplexes to reach at a high knockdown efficiency. Initial studies established basic rules concerning the target selection, such as:

(i) The target region might be selected from the open reading frame, preferably 50 to 100 nt downstream of the start codon.

(ii) 5' or 3' untranslated regions (UTR) must be avoided.

(iii) Preferably mRNA target sequences with 5'-AA(N19)UU motif and those with 50% G/C content should be chosen.

(iv) Highly G-rich sequences should be avoided since they tend to form structures like hairpin.

(v) Additionally, a BLAST (Basic Local Alignment Search Tool) search must be used to ensure that only a single gene is targeted (Elbashir, Harborth et al. 2002). Regarding shRNA stem length, it can vary between 19-29 nt. However, it has been reported that 27-29 nt siRNA are almost 100 times more potent of inducing target mRNA degradation than conventional 21 nt siRNAs (Siolas, Lerner et al. 2005). Additionally, loop size can vary between 4 to 23 nt, and 9 nt loop has proven to be optimal in many applications (Brummelkamp, Bernards et al. 2002).

Further studies added specific rules termed "thermodynamic constraints" regarding special nt within the siRNAs. The objective of these rules is to permit a complete strand separation of siRNAs as a condition for active RISC formation (Schwarz, Hutvagner et al. 2003). Reynolds et al. established that the functionality is determined by specific properties of siRNA and not by the local mRNA target properties. Some of the criteria suggested are: 30-52% G/C content, the presence of three or more A/U bp in positions 15-19 of Sense Strand (SS), lack of internal

repeats, the presence of an A at position 19 of the SS, the presence of a U in position 10 of the SS, the absence of a G or C at position 19 of the SS and a G at position 13 of the SS correlated with a high percentage of target inhibition. This algorithm was useful enhancing the probability of selecting siRNAs with silencing more than 50% (Reynolds, Leake et al. 2004). Similarly, Ui-Tei and colleagues reinforced the search of effective RNAi with additional conditions, for example: A/U at the 5' end of the Antisense strand (AS), AU-richness at the 5' terminal of AS and the absence of any long GC stretch of more than 9 bp in length (Ui-Tei, Naito et al. 2004). Further studies have shown concordance with the previous algorithm. Although a few of them are completely necessary to design siRNAs highly efficient, some of them such as A/U at position 10 and G/C at position 11, showed an increased efficacy (Jagla, Aulner et al. 2005). Despite this, a recent study showed that Reynolds scoring does not provide positive correlative criteria for shRNA design. Significantly, the algorithm of Ui-Tei correlates directly with shRNA efficacy, although it is necessary to establish some modifications concerning thermodynamic features (Taxman, Livingstone et al. 2006).

Special attention has been focused on the dependence of RNAi activity and the structure of the target RNA. The incorporation of some nt targeted in hairpin structures resulted in a considerable reduction of the efficiency in gene silencing (Schubert, Grunweller et al. 2005). Furthermore, some algorithms have been developed to predict the accessibility of the mRNA target, calculating the theoretical maximum stability of a base-paired duplex and determining the most probable secondary structure of the target (Heale, Soifer et al. 2005). However, other studies did not consider that the mRNA target structure could influence the silencing mechanism (Ui-Tei, Naito et al. 2004). Recently, several web servers including algorithms with previously described rules have been developed. Each tool has specific characteristics to perform the search of effective shRNAs; some of them are as follows: (i) Whitehead, combined Tuschl and Reynolds rules, perform BLAST of candidates and the results are sorted by thermodynamic characteristics

(Yuan, Latek et al. 2004); (ii) GenScript, includes a combination of Reynolds and Ui-Tei guidelines (Reynolds, Leake et al. 2004; Ui-Tei, Naito et al. 2004). Also filters target sites based on RNA duplex internal stability and strand secondary structure (Wang and Mu 2004); (iii) SiSearch, presents additional rules-Stockholm rules as Reynolds and Ui-Tei guidelines. The analysis is based on energy features and include BLAST search too (Chalk, Wahlestedt et al. 2004).

Finally, although most algorithms for shRNAs/miRNAs design include all the previous analysis, it is not always probable to find candidates that will knockdown a target gene with 90% efficiency. There are several other factors, such as cellular and molecular factors that could influence the final result of our approach.

1.3.6. siRNA-mediated toxicity and off-targeting. Since the RNAi discovery, numerous studies have demonstrated useful approaches of this mechanism to down-regulate gene expression. One of the principal characteristics for effective RNAi is the perfect complementarity of siRNA and its target mRNA. However, siRNAs can direct RNAi to target mRNA sequences that lack complete sequence identity. Saxena et al reported that a short RNA duplex could silence an endogenous gene with partial complementarity at 3' UTR and the coding sequence region of a gene. Although 3-4 mismatches were introduced into siRNA, it suppressed the expression of the endogenous gene (Saxena, Jonsson et al. 2003). Furthermore, special attention should be kept into account when target specificity is necessary in therapeutics or functional genomic analysis. Also, Jackson et al. found an 8 nt conserved region from siRNAs 5' AS, being the most important region to off-targeting phenotype (Jackson, Bartz et al. 2003). Particularly, the BLAST algorithm search used broadly for siRNA design is not specifically useful to assess RNAi off-target effects. Therefore, it has been proposed computational approaches, which bring information about both in vivo off-target rates and the properties of the siRNA binding/knockdown process (Qiu, Adema et al. 2005). Chemical modifications can reduce off-target effects. siRNAs

can be modified to cause less off-target effects by replacing the 2' OH group with 2'O-methylation, 2'H or 2'F modification in the seed region of siRNA without altering target-specific knockdown (Jackson, Burchard et al. 2006). In addition, G:U pairing at positions 2-8 of the guide strand, eliminates seed-dependent off-target gene silencing without altering gene silencing activity (Ui-Tei, Naito et al. 2008).

On the other hand, a second undesired effect of RNAi is the activation of dsRNA-activated Protein Kinase (PKR)-Interferon (IFN) response that is triggered following dsRNA exposure. In fact, transfection of 21-bp siRNAs duplex resulted in IFN-mediated activation of Jak-Stat signalling pathway. It was suggested an unpleasant effect when siRNAs were introduced into cells, because earliest studies showed that only long RNA duplex, more than 30bp, triggered IFN responses. Notably, the knockdown phenotype resulting from silencing siRNA-dependent could be mimic for this non-specific mechanism of inhibition (Sledz, Holko et al. 2003). In addition, U- and G- rich regions, for example UGUGU, seem to be a common feature among the immunostimulatory motifs of siRNAs. Innate immune activation by siRNA was dependent on the use of a delivery vehicle. In contrast, unmodified naked siRNA did not induce cytokine response at equivalent levels (Judge, Sood et al. 2005). Due to the significance of any adverse effect after RNAi treatment in clinical trial, several attempts to avoid it have been described. Akashi and colleagues showed that shRNA sequences of 50-bp and 100-bp with multiple mutations within the SS, including G:U base pairs, caused efficient suppression of the replication of the Hepatitis C Virus without triggering IFN response (Akashi, Miyagishi et al. 2005). Another advance to avoid this undesired effect was the introduction of 2' -O-Methyl modifications to 5% of guanosines or uridines within one strand of the duplex, so its immunostimulatory activity of siRNA, was fully abrogated (Judge, Bola et al. 2006).

Above all, the challenge to design efficient siRNAs to down-regulate an mRNA target covers multiple issues, which have to be considering together, because each

requirement and characteristic of this system influences the possible outcome phenotype *in vitro* and *in vivo*.

1.4. RNA INTERFERENCE AND HIV-1 INFECTION

1.4.1 Introduction. RNAi has shown to be a powerful technology to suppress gene expression in several infectious diseases. Regarding HIV-1, several studies have shown significant inhibition in virus replication by targeting mRNA of structural and regulatory genes. First studies, used *gag* and *env*-dsRNAs (~400-430 bp), which showed a reduction of p24 protein expression by ~23 fold compared to the untreated control cells in peripheral blood mononuclear cells (PBMC) (Park, Miyano-Kurosaki et al. 2002). Also, transient co-transfections with ssiRNA targeting *gag* and 3' LTR of HIV-1 showed a significant inhibition of viral replication. Notably, this inhibition was due to the cleavage of genomic RNA in the cytoplasm and RNA viral transcripts synthesized from the integrated proviral DNA (Capodici, Kariko et al. 2002). In contrast, Westerhout et al. found that several shRNA against different regions of HIV RNA genome such as *nef*, were unable to inhibit an incoming lentiviral vector genome. It could be explained by the inaccessibility of the genome to the RNAi machinery, due mainly to viral proteins, which are still binding to the reverse transcription complex (Westerhout, ter Brake et al. 2006). Although ssiRNAs showed important RNAi activity, these effects were transient. Additionally, the escape mutant emergence was the most important challenge to overcome, and it still represents an imperative task. Therefore, new approaches are being described constantly; shRNAs under control of the H1 promoter in a retroviral vector, against host cell protein as CCR5 co-receptor, resulted in 68% inhibition of infection with a CCR5-dependent, M-tropic, HIV-1 isolate. Likewise, transient transfection of HeLa CD4 cells with siRev followed by infection with a CXCR4-dependent, T tropic HIV-1 strain resulted in inhibition of viral replication in more than 90%. Similarly, stable transfectants expressing siCCR5 and siRev showed sustained protection close to 90% for more than 2 weeks (Arteaga, Hinkula et al.

2003). In addition, siRNAs targeting CD4 receptor reduced HIV-1 entry of both R5 and X4 virus (Novina, Murray et al. 2002). Notably, siRNAs derived from a lentiviral vector under the U6 promoter combined with an anti-CCR5 ribozyme and a TAR RNA decoy showed a superior inhibition of HIV-1 replication in PBMC and CD34+-derived monocytes (Li, Bauer et al. 2003).

Moreover, targeting HIV-1 highly conserved regions has been suggested as other system to prevent possible escape mutants from the selective pressure of the siRNA inhibition. siRNAs targeting highly conserved genes such as *gag*, *pol*, *integrase*, *vpu* and *env* effectively suppressed HIV-1 replication in chronically infected cells and in PBMC (Chang, Liu et al. 2005). The expression of multiple siRNAs derived from dsRNA and long hairpin RNAs (lhRNAs) of approximately 300 bp driven by inducible promoter, targeting *tat*, *nef* and *rev* reduced for more than 60% the HIV-1 replication without induction of any IFN response. Because a larger segment of the viral genome is targeted, probably the chance of viral escape is reduced (Konstantinova, de Vries et al. 2006). An analysis of 86 shRNA constructs targeting mainly high conserved HIV-1 sequences, -some of them were previously published-, showed a stronger inhibition when two shRNAs were coexpressed than singles shRNAs expression (ter Brake, Konstantinova et al. 2006). Recently, Saayman and colleagues used a panel of shRNAs and lhRNAs expressed by an U6 promoter to generate siRNAs sequences against two separate overlapped reading frames: *tat/rev* and *rev/env* and a unique region within *vif*. Although, some problems in the cleavage of the third siRNA by Dicer were described, the most effective inhibition (~60-70%) was achieved with lhRNA *tat-rev-vif* followed by lhRNA *rev-vif-tat*. (Saayman, Barichievy et al. 2008).

Since, it was demonstrated that ssiRNA was able to down-regulate the expression of an mRNA bearing partially mismatched and miRNAs can induce the cleavage of mRNAs bearing totally complementary target sites (Zeng, Yi et al. 2003), various analyses have included the expression of short hairpins inserted in miRNAs backbones. Boden et al. replaced a part of the miR-30 pre-miRNA stem sequence

transcribed by pol II promoter, with a siRNA targeting HIV-1 *tat*. This siRNA was ~80% more effective than the inhibition for conventional shRNAs (Boden, Pusch et al. 2004). A recent study demonstrated that the simultaneous expression of four anti-HIV siRNAs derived from a miR-17-92 backbone and localized at its 5' end resulted in optimal HIV-1 *gag*, *pol* and *tat* inhibition (Liu, Haasnoot et al. 2008).
However, a clinical assay in mice using shRNAs showed a possible competition between shRNAs and miRNAs resulting in toxicity in the liver and some of the treated mice died. The mechanism suggested of this undesired effect was the saturation of Exportin-5. So, the regulation activity of the miR-122, a miRNA liverspecific, was blocked (Grimm, Streetz et al. 2006). Later on, it was suggested that this effect was due to a competition of siRNAs, shRNAs and miRNAs at the level of incorporation into RISC. Also, it varies significantly between cell lines and correlated with differences in the expression levels of Ago2; an over-expression of Ago2 resulted in an attenuation of competition (Vickers, Lima et al. 2007).

1.4.2 Suppression of RNAi by HIV-1. Soon after the inhibition of HIV-1 replication mediated by siRNAs targeting different viral transcript sequences was demonstrated, a critical obstacle was found. Since the silencing mechanism by siRNAs requires a near fully complementarity with the mRNA target, any mutation at siRNA-target sequence represents a complete resistance to RNAi activity. Boden et al were one of the first researches to report viral escape mutants and described its probable cause. They showed that synthetic siRNAs targeting *tat* reduced HIV-1 replication by 95% in stably transfected cells. However, three weeks later, viral species emerged with a non-synonymous mutation at 9 nt enabling viral replication (Boden, Pusch et al. 2003). Also, transduced cells with siRNA against *nef* showed a significant HIV-1 inhibition for more than 8 months, but a deletion of 106 bp within siRNA-*nef* target facilitated selection of HIV-1 escape mutants. In addition, nucleotides substitutions were reported. Significantly, the deletions did not affect essential regulatory elements such as the 3' polypurine tract or LTR overlapped sequence (Das, Brummelkamp et al. 2004). Nevertheless, more

analyses on the cause of siRNA resistance, illustrated single nt substitutions which changed the secondary structure of RNA target, resulted in an occlusion of the target sequence in a stable hairpin structure. In fact, most nt substitutions were observed in the central and 3' region of the target affecting RNAi efficiency (Westerhout, Ooms et al. 2005). Additionally, an important finding demonstrated that HIV-1 Tat protein is a suppressor of RNA silencing (SRS) as a manner to combat nucleic acid-based immunity by HIV-1 in human cells (Bennasser, Le et al. 2005). Tat created a direct suppression of Dicer processing activity in RNAi by interaction with a portion at 30-72 amino acids of Tat and RNA (Bennasser and Jeang 2006). The authors also showed that TAR (trans-activation responsive RNA) HIV-1 is used to attenuate antiviral RNAi by sequestration of TRBP, a key component of Dicer-RISC complexes, furthermore blocking RNA silencing pathway (Bennasser, Yeung et al. 2006).

1.4.3 *nef* and siRNA therapy. The first approach of RNAi against *nef* was performed using synthetic dsRNA targeting *nef* of some LTNPs as well as F12 *nef*. The latter, is a *nef* allele mutated whose expression in stably transfected cells fails to downregulate the CD4 receptor and induces an antiviral state. Both RNA duplexes inhibited the expression of a fusion reporter protein and HIV-1 replication as well (Yamamoto, Omoto et al. 2002). Also, synthetic siRNA or plasmid-derived shRNAs directed to LTR, *vif* and *nef* specifically reduced virus production by degradation of HIV-1 genome (Jacque, Triques et al. 2002). Likewise, RNAi duplexes against *nef* and *gag* genes showed a inhibition on neurotropic strains of HIV-1 (Dave and Pomerantz 2004). However, the latest siRNA*nef* did not show RNAi activity in an analysis carried out with 86 shRNA constructs targeting highly conserved HIV-1 sequences (ter Brake, Konstantinova et al. 2006). Recently, Yamamoto and colleagues reported a inhibition over 90% of *nef* mRNA expression by shRNAs derived from a lentivirus vector targeting U3-overlapping region of HIV-1 *nef* in a monocytic cell line and primary monocyte-derived macrophages (Yamamoto, Miyoshi et al. 2006).

Particularly, a new approach has been suggested to avoid possible escape variants. It is based on designing shRNAs, used as a second challenge, against expected escape targets, thus reducing the chance of RNAi viral resistance. It was named second generation of shRNAs. Notably, single *nef*-nt mutants were potently inhibited with these shRNAs. However, it was not functional for escape mutants with a modified RNA secondary structure (ter Brake and Berkhout 2005).

On the other hand, it has been demonstrated by computer-directed analyses that HIV-1 uncovered five pre-miRNAs candidates localized in different regions of HIV-1 genome: close TAR, *gag*, near the *gag-pol* frameshift, in the *nef* gene and in the 3' LTR. These probably produced in 10 viral miRNAs (vmiRNA). Also, it was suggested that each viral miRNA could target ~50 to 100 cellular RNAs, which include a large amount of proteins such as major histocompatibility complex class II, 2'-5' oligoadenylate synthetase, macrophage colony-stimulating factor, p21-activated protein kinase Pak2, etc implicated in diverse cellular processes (Bennasser, Le et al. 2004). Notably, Omoto and colleagues reported *nef*-derived miRNAs in HIV-1 infected cells. In fact, these miRNAs inhibited Nef-EGFP expression (Omoto, Ito et al. 2004). Remarkably, it was showed that one the previous *nef*-miRNAs (miR-N367) suppressed HIV-1 LTR promoter activity. The target sequences was localized within the U3 region of the 5' LTR and *nef* sequence overlapped (Omoto and Fujii 2005).

Finally, several studies have shown highly efficient inhibition of different HIV-1 genes by using either synthetic siRNA or plasmid encoded shRNA. However, given to the high mutation rate of the HIV-1 genome viral escape mutants are rapidly induced. Essential proteins for viral replication should work at a wide extent of mutated variants and their encoding-DNA sequences might be under a high selection pressure due to essential functions of these proteins. Conversely, proteins conferring viral virulence, but not essential for replication, when targeted by siRNA, could induce low virulence escape mutants which are under lower selective pressure. Strategic selection of targets, i. e., sequences coding for highly

conserved and functional domains, may attenuate this phenomenon but eventually produce disable mutated HIV-1 strains. Remarkably, Nef is a viral virulence protein, but dispensable for replication. This protein is expressed early in HIV-1 infection and has several fucntions that explain in part its role as a virulence factor. It has been reported that Nef is involved in the process of down-regulating several surface receptors, as CD4 and MHC class I. Nef also interacts with protein kinases at different signaling pathways and is involved in events of apoptosis down-regulation. Moreover, some deletions and single mutations in *nef* have been found in Long Term non-progressors suggesting that viral strains lacking Nef or harboring non-fuctional variants of this protein has a low virulence conferring to the infected patients long term of survival free of disease. Therefore, Nef has been chosen as a model to prove the main hypothesis of this work.

2 AIMS OF THE THESIS

2.1. MAIN AIM

Finding and evaluation of small interfering RNA (siRNA) sequences with efficient RNA interference (RNAi) activity against strategic regions of HIV-1 *nef* gene.

2.1. SPECIFIC AIMS

(i) Selection and identification of target regions in the *nef* gene of HIV-1 for siRNA intervention

(ii) Design of siRNA sequences with high probability to efficient RNAi function

(iii) Evaluation of the siRNA candidate sequences using diverse gene transfer technologies

(iv) Generation of HIV-1 susceptible cell lines stably expressing the efficient siRNAs against HIV-1.

3 MATERIALS AND METHODS

3.1. siRNA SELECTION, DESIGN AND SHORT-HAIRPIN RNA EXPRESSION VECTORS CONSTRUCTION.

The sequence selected as target is *nef* HIV-1 gene sequences from HXB2 strain-GenBank accession number K03455, from 8797 to 9417 nt (621 pb). The sequences were chosen according mainly two criteria: (i) conservation with respect to a consensus sequence and (ii) functionality of the protein domains coded by the target sequence. The HIV Sequence alignments tool on HIV sequence Database from Los Alamos National laboratory was used in order to analyze highly conserved sequences on both nucleotide and protein level (Leitner 2006).

Based on Reynolds (Reynolds, Leake et al. 2004) and Ui-Tei (Ui-Tei, Naito et al. 2006) rules, were designed by hand nine shRNA sequences, which are included into high conserved and essential *nef* region. As a negative control, one shRNA was also designed with two main criteria's using the siSearch software tool (Chalk, Wahlestedt et al. 2004). As a positive control of *nef* down-regulation, a sequence from a *nef*-derived miRNA encoded for HIV-1 (miR-N367) that has been able to block HIV-1 Nef expression in vitro, was used (Omoto, Ito et al. 2004). Every sequence was checked with the BLAST program, in order to verify the target sequence. In addition, to minimize potential off-target silencing effects, only sequences with more than three mismatches against unrelated sequences were selected (Semizarov, Frost et al. 2003).

The sequences were designed as 64 nt templates which included the BglII and HindIII restriction sites at the 5' and 3' end respectively, the 19 nt sense siRNA sequence (which in turn corresponds to a 19-nt sequence within the mRNA); a loop

of 9 nt and the 19 nt target sequence in antisense direction as was described previously (Brummelkamp, Bernards et al. 2002). The localization of the selected target sequence on *nef* ORF and the corresponding domains and amino acid residues at the Nef protein were as follows: (i) shMyrSig1 : Myristoylation signal_1 5' TGGGTGGCAAGTGGTCAAA 3'. (ii) shMyrSig2: Myristoylation signal_2 5' ATGGGTGGCAAGTGGTCAA 3'. (iii) shPxxP3: PxxP motif-SH3 binding site_3 5' GTCACACCTCAGGTACCTT 3'. (iv) shPxxP4: PxxP motif-SH3 binding site_4 5' CACACCTCAGGTACCTTTA 3'. (v) shPPT5: Polypurine tract-PPT_5 5' GGGACTGGAAGGGCTAATT 3'. (vi) shPPT6: Polypurine tract-PPT_6 5' GGGGGACTGGAAGGGCTAA 3'. (vii) shPPT7: Polypurine tract-PPT_7 5' AAAGGGGGGACTGGAAGGG 3'. (viii) shNefmiR: Nef–miRNA (miR-N367) 5' CTGACCTTTGGATGGTGCT 3'. (ix) shDimer1: Dimerization region_1 5' CCCTGATTGGCAGAACTAC 3'. (x) shDimer2: Dimerization region_2 5' CCTGATTGGCAGAACTACA 3'. (xi) shNC: Negative control 5' GCATCTCGAGACCTGGAAA 3'. Table 1 shows the 64 mer DNA oligos listed, the sequences in bold shown target sense and antisense sequences. The termination signal of transcription is written on cursive.

These sequences were obtained from The Midland Certified Reagent Company (USA) in 0.05 µM scale. The vector pSuper.retro.puro.stuffer version® was purchased from Oligoengine company (USA). The forward and reverse 64 mer DNA oligos were annealing with annealing buffer: 100mM NaCl and 50 mM HEPES pH 7.4. The mixture was incubated at 90℃ for 4 min, then at 70℃ for 10 minutes and slowly cooling to 10℃. The pSuper.retro.puro stuffer version vector was linearized with BglII and HindIII (New England Lab) and purified from agarose gel (QIAquick® Gel extraction kit, QIAGEN). The oligos were cloned into the vector. The recombinant pSUPER.retro vector was transformed into DH5α E.coli according to the transformation protocol routinely used in our laboratory and the bacteria were grown in ampicillin-LB plates overnight. Thereafter, the colonies were picked and grown in an ampicillin broth for an additional cycle. Several DNA

extractions were prepared using a commercial kit (QIAprep® Spin miniprep kit, QIAGEN) and some colonies were screened with EcoRI and HindIII. A positive colony will release a fragment of 281 bp. In addition, all pSUPER.retroshRNA vectors were verified by DNA sequencing (CORPOGEN) to check the correctness of shRNA oligos and their matching with mRNA*nef* target. Two previously designed and tested shRNA-encoding plasmids, pSRshRNAGFP and pSRshRNARev, were used as positive and negative control of interference activity, respectively (Arteaga, Hinkula et al. 2003).

Table 1. 64 mer DNA oligos designed for each shRNA sequence against *nef*.

shRNAs against Nef	DNA oligos sequence	Localization on *nef*-HXB2	Amino acid residues on Nef-HXB2
shMyrSig 1	5´GATCCCC**TGGGTGGCAAGTGGTCAAA**ttcaagaga**TTTGACCACTTGCCACCCA***TTTTT*GGAAA3'	2-20	1-7
shMyrSig 2	5´ GATCCCC**ATGGGTGGCAAGTGGTCAA**ttcaagaga**TTGACCACTTGCCACCCAT***TTTTT*GGAAA3´	1-19	1-6
shPxxP3	5´ GATCCCC**GTCACACCTCAGGTACCTT**ttcaagaga**AAGGTACCTGAGGTGTGAC***TTTTT*GGAAA3´	208-226	69-75
shPxxP4	5´GATCCCC**CACACCTCAGGTACCTTTA**ttcaagaga**TAAAGGTACCTGAGGTGTG***TTTTT*GGAAA3´	210-228	70-76
shPPT5	5´GATCCCC**GGGACTGGAAGGGCTAATT**ttcaagaga**AATTAGCCCTTCCAGTCCC***TTTTT*GGAAA3´	285-303	95-101
shPPT6	5'GATCCCC**GGGGGACTGGAAGGGCTAA**ttcaagaga**TTAGCCCTTCCAGTCCCCC***TTTTT*GGAAA3´	283-301	94-100
shPPT7	5´GATCCCC**AAAGGGGGGACTGGAAGGG**ttcaagaga**CCCTTCCAGTCCCCCCTTT***TTTTT*GGAAA3´	279-297	93-99
shNefmiR	5'GATCCCCCTGACCTTTGGATGGTGCTttcaagaga AGCACCATCCAAAGGTCAG TTTTTGGAAA3´	409-427	136-142
shDimer1	5´GATCCCC**CCCTGATTGGCAGAACTAC**ttcaagaga**GTAGTTCTGCCAATCAGGG***TTTTT*GGAAA3´	363-381	121-127
shDimer2	5´GATCCCC**CCTGATTGGCAGAACTACA**ttcaagaga**TGTAGTTCTGCCAATCAGG***TTTTT*GGAAA3´	364-382	121-127
shNC	5´GATCCCC**GCATCTCGAGACCTGGAAA**ttcaagaga**TTTCCAGGTCTCGAGATGC***TTTTT*GGAAA3´	96-114	32-38

3.2. REPORTER VECTORS CONSTRUCTION.

3.2.1 Generation of Nef-d2EGFP fusion construct. The pNef-d2EGFPN3 construct was generated by cloning a full-*nef* coding, amplified by PCR and placed at the 5' end of *dEGFP* gene into pd2EGFP-1 vector (BD Biosciences Clontech®). Firstly, the entire *nef* cDNA was prepared by PCR amplification from pCMXnef vector, gently donated from Cristopher Aiken (Aiken, Konner et al. 1994). The pCMXNef vector contains a full-length *nef* coding region derived from the HIV1 HXB2D isolate. *nef* was amplified by PCR, using the oligonucleotide primer 5' TAGGAATTC**ATGGGTGGCAAGTGGTCAAAAAG** 3', carrying an EcoRI site (underlined) positioned upstream of N-terminal *nef* sequence (in bold) and a reverse primer 5' AGTGTCGAC**GCAGTTCTTGAAGTACTCCGGAT** 3', carrying the BamHI site (underlined) downstream of C-terminal sequence of Nef after removal of the stop codon. The primer sequences were purchased from The Midland Certified Reagent Company (USA) in 0.05 µM scale. Amplification was performed in 30 cycles with 0,5 µM in each primer, 0.2 µg of pCMXNef, 200 µM of the four dNTPs, 1X Buffer, 1.5 mM $MgCl_2$ and 0,025 U of Ampli Taq Gold Polymerase (Applied Biosytems). Each cycle consisted of denaturation for 30 seconds at 94ºC, annealing for 30 seconds at 59ºC, and elongation for 1 minute at 72ºC. Th e denaturation time was lengthened to 6 min inthe first cycle and the elongation time to 10 min in the last cycle. The length of the product was 628 bp.

The PCR product obtained was purified by QIAquick PCR purification kit® (QIAGEN), then digested with EcoRI and Sal I restriction enzymes (New England Lab®) at 37ºC for 2 hours. The pd2EGFP-1 (BD Biosciences Clontech®) vector was also digested by EcoRI/Sal I at the same temperature and time conditions as well to generate pNef-d2EGFP-1. The vectors digested were running in an 1.2% agarose gel, 90 V for 1 hour, cut and purified from gel slice by QIAquick Gel Extraction kit ® (QIAGEN). The ligation reaction was incubated at 16ºC for 12 hours, using T4 DNA ligase (Invitrogen®). The transformation was done in *Escherichia coli* JM109 strain and positive clones were grown in Kanamycin LB

plates. The positive colonies were screening with EcoRI/Sal I digestion to verify if the *nef* segment (628 bp) was cloned.

The half-life of dEGFP is approximately 2h, a very rapid protein turnover compared with normal EGFP, which is 24h (Li, Zhao et al. 1998). For this reason, the destabilized Green Fluorescent Protein was chosen as the reporter protein of siRNA effect.

Note: pd2EGFP-1 is a promoterless d2EGFP vector. Promoter/enhancer elements should be inserted into the MCS upstream of the d2EGFP coding sequences. Therefore, the *nef-d2EGFP* segment (1531 bp) already cloned into the previous vector was cloned into pEGFPN3 expresion vector (BD Biosciences Clontech®). Both vectors pNef-d2EGFP-1 and pEGFPN3 were digested by SacI/Not I, purified from slice agarose with QIAquick Gel Extraction kit ® and ligated with T4 DNA ligase (Invitrogen®). The transformation was done in E. coli JM109 strain and positive clones were grown on Kanamycin LB plates. The positive colonies were screened with Sac I/Not I digestion in order to verify if *nef-d2EGFP* segment (1531 bp) was cloned. The final reporter vector with fusion protein Nef-d2EGFPN3 is 5480 bp. The fusion protein expression is driven by Human Cytomegalovirus (CMV) promoter.

3.2.2 Generation of pd2EGFP construct. One more vector expressing only d2EGFP was cloned. The pd2EGFP-1 and pEGFPN3 expression vector were digested with BamHI/NotI. Both digestions were purified from slice gel with QIAquick Gel Extraction kit ® and ligated with T4 DNA ligase (Invitrogen®). The transformation was done in E. coli JM109 strain and positive clones were grown on Kanamycin LB plates. The positive colonies were screened with BamHI/Not I digestion in order to verify if *d2EGFP* segment (869 bp) was cloned. The final reporter vector expressing d2EGFP is 4862 bp. The protein expression is driven by Human Cytomegalovirus CMV promoter.

Both pNef-d2EGFPN3 and pd2EGFPN3 reporter vectors were sequenced to check the correctness of the fusion protein and verify the right position of these genes.

3. 3. CELL CULTURE AND TRANSFECTION.

HeLaT4 cells (Human cervix carcinoma cell line) express constitutively CD4 on their surface (Miller and Rosman 1989) were used for transfection. They were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, in an atmosphere of 5% CO_2 at 37℃. The DMEM was additionally supplemented with 0.5 mg of G418/ml. The cells were transfected at approximately 40–50% confluency using both linear polyethylenimine (l-PEI) and Fugene-6 (Roche) according to the manufacturer's instructions. In brief, cells were seeded onto 6-well plates and the next day transfected using a ratio of 1:3 (reporter vector either 1 µg pd2EGFPN3 or pNef-d2EGPFN3: 2µg psuper.retro.shRNA). The cells were incubated at 37℃, 5% CO_2. 24h later the medium was replaced by fresh one. The cells were routinely analyzed 48 h post-transfections (Choi, Cho et al. 2005). All the transfections were assessed by epifluorescence microscopy and the average of transfection efficiency was 70-80%.

3.4. CONFOCAL MICROSCOPY.

Hela-T4 cells were cultured in 6 well dishes for 18h. Thereafter, the cells were transfected with 2µg plasmids expressing shRNA and 1 µg of the pNef-d2EGFP with 6 µl of Fu-Gene (Roche) following the recommendations of the manufacturer. After 48 h of transfection, the cells were washed with PBS and fixed with 3% paraformaldehyde for 12 min. Cells were washed three times with PBS and incubated for 15 min in a blocking buffer containing 0.1% Triton X 100 (St Louis), and 0.1% of BSA-C in PBS. The cells were then rinsed 5 min with PBS, and

counterstained with a dilution of 1:3000 of wheat-germ agglutinin, conjugated with tetramethylrhodamine (Molecular Probes BV) and 1:4000 of Hoechst (Fluka Chemic AG) in PBS, for 10 min in darkness. Finally the cells were washed four times for 5 min with PBS and visualized by fluorescence microscopy with a Leica DMRXA microscope (Leica Microsystems Gmbh, Wetzlar, Germany) equipped with a CCD camera (Model S/N 370 KL 0565, Cooke Corporation). Filter sets for DAPI/ Hoechst, Cy3 and Cy5 were obtained from Chroma technology (Brattleboro). The images were acquired using the Slidebook 2.1 software (Intelligent Imaging Innovations Inc) (All these procedures were performed at Karolinska Institutet facilities).

3.5. FLOW CYTOMETRY.

Hela-T4 cells were cotransfected with 2µg of the plasmids expressing shRNA and 1 µg of the pNef-d2EGFP with the Fu-Gene transfection reagent following the recommendations of the manufacturer (Roche). After 48 hrs of transfection, the percentage of GFP positive cells was quantified by FACS analysis using a BD FACSCalibur system with filters (emission, 507 nm; excitation, 488 nm) for EGFP expression. Samples were counted, and analyzed. Non-transfected HeLaT4 cells were used as control (at Karolinska Institutet facilities). The average of the GFP intensity level was estimated for each shRNA and compared with the GFP expression level of shRNARev.

3.6. SDS-PAGE ELECTROPHORESIS AND WESTERN BLOT ANALYSIS.

HeLa T4 cells were trypsinized 48 h post-transfections, washed once with ice cold PBS 1X and pelleted by centrifugation at 1500 rpm, 4℃ for 10 min. The cells were lysed on ice for 15-20 min in a proper RIPA buffer volume including 150 mM NaCl, 4% (w/v) 3-[(-cholamidopropyl)dimethylammonio]-1-propanelsufonate hydrate

(CHAPS) (ALDRICH), 0.5% deoxicolato de Sodio (SIGMA), 0.1% Sodium Dodecyl sulfate -SDS (SIGMA), 50mM Tris-HCl pH 8.0 and protease inhibitor cocktail (SIGMA). The samples were mixing by vortexing and the cell debris was removed from cell extract by centrifugation at 13.000 rpm, 4℃ for 10 min. The total proteins in the lysate were determined using Quick Start Bradford protein Assay ®(Biorad) with BSA standard sets.

Cleared lysates containing ~35 µg of total protein on Laemmli buffer 2X were resolved by SDS-PAGE with 12% acrylamide (Biorad) in a running buffer containing 24.7 mM TrisBase, 1.44% Glycine and 0.1% SDS. The gel quality was assessed with Coomassie Brilliant Blue R-250 dye (Biorad). Proteins were transferred to Amersham Hybond™ ECL Nitrocellulose membrane of 0.20 µm pore size in a buffer containing 25mM TrisBase, 192mM Glycine and 15% methanol for 1 hour, 100V. The transfer process was assessed with the reversible dye Ponceau S (0,1% w/v) (MERCK). The membranes were blocked in 5% non-fat dry milk in PBS-T buffer (1.37 M NaCl, 27 mM KCl, 43 mM $Na_2HPO_4.7\ H_2O$, 14 mM KH_2PO_4 and 0.05% Tween20) for 2 hours at room temperature, washed with PBS-T several times for 30 min and then incubated overnight at 4°C with a 1:4000 dilution of anti-GFP rabbit polyclonal serum (Invitrogen), The membranes were then washed several times with PBS-T, incubated with Polyclonal Goat anti-Rabbit immunoglobulins/ Horseradish peroxidase(HRP) (Dako cytomation) at a dilution of 1:2000 for 1.5h at room temperature. Detection was performed by SuperSignal® West Pico Chemiluminescent substrate (Pierce) according to the manufacturer's instructions. The blot was exposed to X-ray film.

The blot was washed several times with PBS-T and stripped to remove previous antibodies, using Stripping Buffer (2%SDS (v/v), 100mM β-Mercaptoethanol and 50 mM Tris-HCl pH 6,8) for 15 min at 50℃. Then, the blot was rinsed intensively with PBS-T and blocked in 5% non-fat dry milk in PBS-T buffer for 2 hours at room temperature, washed with PBS-T several times for 30 min and then incubated overnight at 4℃ with a 1:10,000 dilution of of anti-Mouse monoclonal Anti-β-Actin clone AC-15 (SIGMA), The membranes were washed several times with PBS-T,

incubated with Polyclonal Goat anti-Mouse immunoglobulins/ Horseradish peroxidase(HRP) (Dako cytomation) and used at 1:2000 dilution for 1.5h at room temperature. Detection was performed by SuperSignal® West Pico Chemiluminescent substrate (Pierce) according to the manufacturer's instructions. The blot was exposed to X-ray film.

3.7. RNA ISOLATION, cDNA SYNTHESIS AND QUANTITATIVE REAL-TIME PCR

Total RNA was isolated 48h after cotransfections in HeLa T4 cells, using SV Total RNA isolation System (Promega, Madison) and TRizol LS (Invitrogen, USA) according to the manufacturer's instructions. The starting cell number was approximately $1x10^6$ cells and $2x10^6$ cells for each method, respectively. The yield and purity of total RNA was obtained by spectrophometry at 260nm (UV Genesys 10 analyzer), relative absorbances at 230, 260 and 280 nm. The ratio A_{260}/A_{280} was ranged within 1.7-2.1. The integrity of the purified RNA was assessed by agarose gel electrophoresis.

The total RNA was treated with DNase RQ1 (Promega). Then, the concentration of the RNA was determined by spectrophotometry at 260 nm, where 1 absorbance unit (A260) equals 40µg of single-stranded RNA/ml.

Total RNA was converted into cDNA using a first strand cDNA synthesis kit (Fermentas) according to the manufacturer's protocol. DNase-treated (1 µg) was added to a reverse transcription (RT) reaction containing M-MuLV Reverse transcriptase (20u/µl), Ribolock™ RNase inhibitor (20u/ µl), 1mM of each dNTP, 1X reaction buffer and 5µM Oligo(dT)18 primers. In addition, NTC and RT- controls were included. Reverse transcription was performed at 37℃ for 1 h followed by heat-inactivation of the RT enzyme at 70℃ for 5 m in. An RNA positive control for GAPDH was performed to assess cDNA synthesis by PCR amplification.

Undiluted-cDNA (2 µl) was added to 18 µl of SYBR® GreenER™ qPCR Supermix Universal (Invitrogen) containing hot-start Taq DNA polymerase, SYBR® GreenER™ fluorescent dye, $MgCl_2$, dNTPs (with dUTP instead of dTTP), UDG and 200 nM of sense primer *nef* 5'AATGCTGATTGTGCCT '3 and antisense primer *nef* 5' GTGTAGTTCTGCCAATC '3; product 230 bp. For RNA normalization, a GAPDH-qPCR was performed with the same PCR reagents except for the primers which were GAPDH sense primer 5' CATGAGAAGTATGACAACAGCCT '3 and GAPDH antisense primer 5' AGTCCTTCCACGATACCAAAGT '3; product 113 bp. Additionally, HPRT primers were designed, in order to test which housekeeping gene was the most suitable according some previous analysis (Watson, Mercier et al. 2007). GAPDH was the housekeeping with an accurate normalization. The primers were designed using Perlprimer open-source (Marshall 2004; Watson, Mercier et al. 2007)**.** The used protocol for *nef*-qRTPCR was: 2 min at 50℃, 10 min at 95℃, 30 s at 95℃, 30 s at 51℃, 1 m at 72° C, plate read for 40 cycles and melting curve from 40℃ to 100℃. The protocol for GAPDH-internal control was performed identical, except for annealing temperature 30 s at 58℃.

The samples of negative control (HeLa T4 untrasfected), scramble control (HeLaT4 transfected pNef-d2EGFPN3+pSRshRNARev), knockdown positive control (HeLaT4 transfected pNef-d2EGFPN3+pSRshRNAGFP) and 11 samples (HeLaT4 transfected pNef-d2EGFPN3+pSRshRNAs against nef) were amplified per triplicate in low-profile 0.2 ml tubes strips of 8 vessel clear (Biorad) in an DNA Engine Opticon® System (MJ Research). To check the amplification product the samples were run on an agarose gel electrophoresis and a melting curve was performed after all cycles.

3.7.1 PCR efficiency and relative expression ratio determination. The raw (i.e. not baseline-corrected) PCR data were used in the analysis. The PCR efficiency for each individual sample was derived from the slope of the regression line fitted to a subset of baseline-corrected data points in the log-linear phase using LinRegPCR software (Ramakers, Ruijter et al. 2003).The relative expression ratio

of *nef* gene relative to a GAPDH gen in shRNAs against *nef* treated samples, was calculated using a formula with individual efficiencies for each gene described by Schefe J et al (Schefe, Lehmann et al. 2006).

4 RESULTS

4.1. FOUR DOMAINS OF NEF ARE HIGHLY CONSERVED AND ESSENTIAL FOR THE PROTEIN FUNCTION; *nef*-CODING SEQUENCES FOR THESE RESIDUES ARE PROPOSED AS ANTI HIV-1 siRNA STRATEGIC TARGETS

It is clear that siRNA against different HIV-1 transcripts have a high inhibitory effect on viral infection and replication. However in several experimental models it has been shown that HIV-1 siRNA escape variants are rapidly selected. To avoid the appearance of resistant strains, viral genome sequences coding for domains that are highly conserved and essential for the function of crucial viral proteins could be strategic targets. Nef sequence shows either single amino acids or motifs highly conserved (Shugars, Smith et al. 1993) playing key roles on HIV-1 infectivity by being essential regions for interactions with host cell proteins implicated in some signaling pathways and down-regulation of CD4 receptor and MHC class I. Here, Nef coding sequences fitting these requirements were identified and selected as strategic siRNA target sequences.

By searching in the literature for data describing structure, function and biochemistry of Nef, highly conserved residue sequences of the protein were identified and from them, those directly involved in functional activities and/or having structural complexity were selected. In addition, for selection of strategic siRNA target regions on the *nef* gene coding sequences, other molecular biology aspects, such as coding for more than one ORF, were considered. At the same time, the HIV Sequence Alignments Tool, available at the HIV sequence Database from Los Alamos National laboratory, was used in order to find highly conserved sequences at both nucleotide and protein level (Leitner 2006). Eight functionally

important and, most of them, conserved domains were identified in the protein. The domain residues and its functional importance are listed in Table 2.

Table 2. Summary of Nef protein residues, essential functions and importance for HIV-1 infection.

RESIDUES	*FUNCTION*	*IMPORTANCE*
R105, D108, I109, L112, Y115, H116, **F121, P122, and D123*** (Shugars, Smith et al. 1993)	Oligomerization	Efficient interaction with a subset of cellular targets (Arold, Hoh et al. 2000). Enhancement of infectivity (Geyer and Peterlin 2001).
1-7 MGGKWSK* (Geyer, Munte et al. 1999).	Myristoylation	Highly conserved (Shugars, Smith et al. 1993). CD4 downmodulation and enhanced viral infectivity. Targeting of Nef to the cytosolic membrane (Geyer, Munte et al. 1999). MHC-I downregulation (Peng and Robert-Guroff 2001).
69-78 P69xxPxxPxxP78 (PVRPQVPLRP)* (Saksela, Cheng et al. 1995).	Binding to SH3 domains of Serine kinases	Highly conserved (Shugars, Smith et al. 1993). Induction of cellular activation (Saksela, Cheng et al. 1995). Class I MHC downregulation (Greenberg, Iafrate et al. 1998).
4-7 KWSK 17-22 RERMRR	Virion incorporation of Nef, and infectivity	Mutations in Met 20 cause loss of the Nef ability to modulate MHC-I expression but not CD4 downmodulation (Akari, Arold et al. 2000).
W57-W58 60-165 EDXXLL 174-179 DDPERE 154-155 EE 174-175 DD	Protease cleavage site (Freund, Kellner et al. 1994). Binding to adaptor complexes AP-1 and AP-2 (Pandori, Craig et al. 1998). β COP(Piguet, Gu et al. 1999).	Downmodulation of CD4 (Grzesiek, Stahl et al. 1996). Enhancement of infectivity (Geyer and Peterlin 2001; Fackler, Moris et al. 2006).
62-65 EEEE	PACS-1	HMC I downmodulation. Highly conserved (Piguet, Gu et al. 1999)
105-106 RR. P69,L76,L112 and F121.	Interaction with Pak2	Signaling and cellular activation (Geyer and Peterlin 2001).
91-96 L K E K G G L E G L I *	Polypurine tract-PPT. Reverse Transcription and integration of viral genome into host cell	Its deletion significantly impaired replication kinetics. The plus-strand synthesis is not completed (Rausch and Le Grice 2004).

*regions of Nef identified as strategic siRNA targets

Therefore, by combining these two types of information, the following four amino acid sequences were identified as highly conserved and essential for Nef function: (1) Myristoylation signal, (2) PxxP motif-SH3 binding site, (3) Polypurine tract-PPT and (4) dimerization region. As shown in Figure 8, an alignment with Nef nucleotide sequences was also performed in order to analyze the conserved region at nucleotide level. Conserved domains of The PxxP motif-SH3 binding site and part of the PPT displayed a high structural complexity, based mainly on the structure and folding of several α helices. The other two conserved regions have a simple, almost linear, structure. The two types of conserved motifs would be used for comparison of role of the structural complexity on the frequency of selection of siRNA-induced escape mutants (Figure 8).

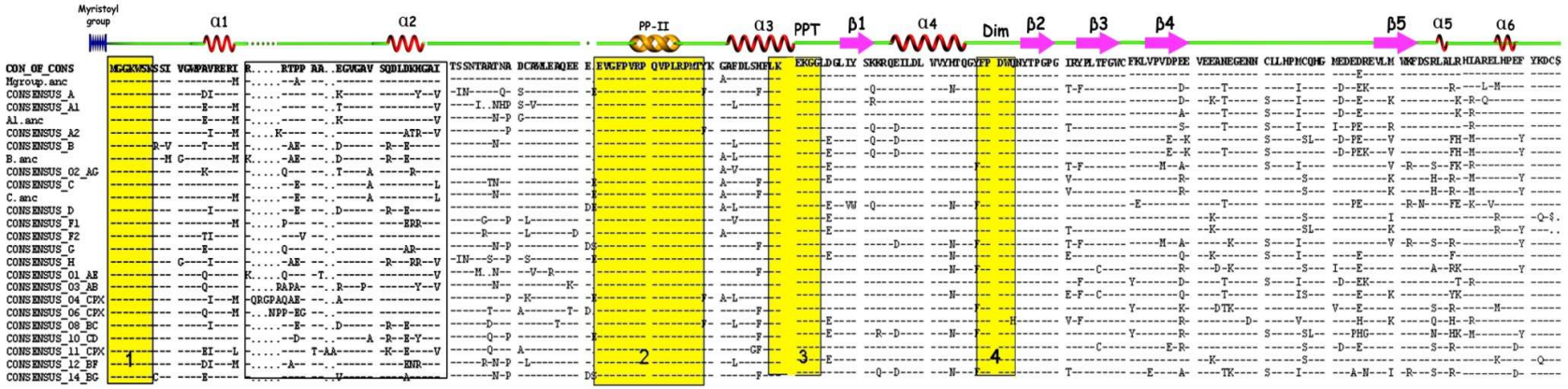


Figure 8. Alignment data with Nef amino acid sequences from consensus/ancestral of HIV-1 M group. The sequences less variable were limited by dark lines and yellow-labeled. The amino acid sequences chosen as highly conserved and essential are: (1) Myristoylation signal, (2) PxxP motif-SH3 binding site, (3) Polypurine tract-PPT and (4) dimerization region. Likewise, an alignment with nef nucleotide sequences was performed in order to analyze the region conserved at nucleotide. Also, the alpha helices, β sheets and important features of Nef structure are point out.

4.2. DESIGN AND CONSTRUCTION OF shRNA SEQUENCES AGAINST STRATEGICALLY SELECTED *nef* HIV-1 TARGET SEQUENCES

From the four identified regions of Nef, as strategic siRNA targets, appropriated RNA complementary sequences were analyzed and selected to design the corresponding shRNA.

Most researchers have used one of the various available algorithms for selecting of siRNAs or shRNAs. The most commonly used ones are: (i) Reynolds (Reynolds, Leake et al. 2004), (ii) UiTei (Ui-Tei, Naito et al. 2004) and/or (iii) Tuschl rules (Elbashir, Harborth et al. 2002). Although these rules may increase the chance of finding effective shRNAs, they will also likely exclude some potentially effective shRNAs that do not follow strictly some of the rules. Due to the restrictions for choosing potential targets present into the four regions of interests that fit the requirements of the above mentioned algorithms, were designed – by hand- nine shRNAs against the conserved and functional regions, using the different rules just as guidelines. To increase the probability of generating efficient shRNAs, as it was possible, more than one shRNA was designed against each region of interest. Some of the shRNA against the same region included overlapping nucleotide sequences.

One shRNA against a region that does not have the already defined criteria for strategic targets, designated as negative control, was designed by using the siSearch software tool (Chalk, Wahlestedt et al. 2004).

Finally, Omoto S et al. have identified a HIV-1 miRNA-*nef* encoded in HIV-1 infected cells: miR-N367. It efficiently down-regulates viral transcription and replication (Omoto and Fujii 2005). Therefore, this miRNA sequence was selected used as a positive control of siRNA mediated inhibition of *nef* expression. In conclusion, the candidate shRNAs were evaluated by BLAST search, to find out of

target complementary sequences. Thus, 11 shRNA sequences were chosen and designed according to the methods described previously.

The commercial Super.retro.puro® system provides a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts, which contain two 3' overhanging nucleotides, such as T or U. The vector uses the polymerase-III H1-RNA gene promoter, as it produces small RNA transcripts lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal, consisting of five thymidines in a row (T5). In this way, stable expression of siRNAs and persistent suppression of gene expression can be achieved (Figure 9).

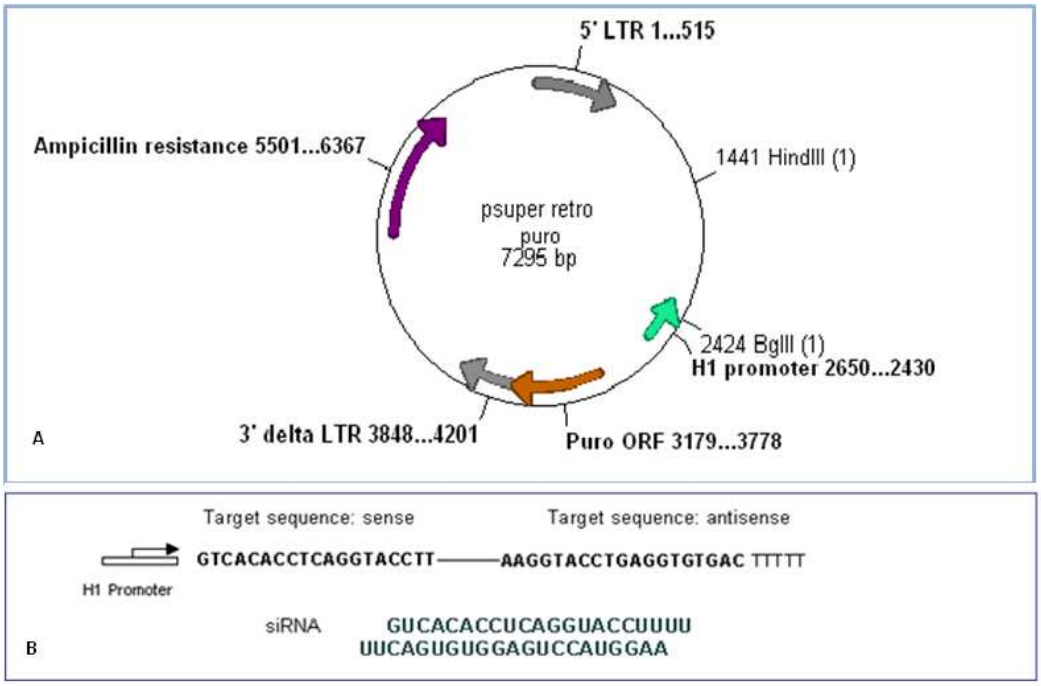


Figure 9. The p.Super.retro-shRNA vector. Design and construction of expression shRNA sequences. All 11 shRNA sequences against nef gene were designed following a design previously tested. A) H1 promoter and restriction enzymes sites are localized in a vector map from ApE –A plasmid editor program (Davis 2006). B) The transcription of 64-nt oligo to hairpin RNA generates functional siRNA of 19 nt with UU overhangs at 3' termini.

The eleven shRNAs homologous to the target mRNA were designed and cloned into a retroviral vector-pSuper.Retro-. The sequences were designated as follows:

(i) Myristoylation signal: 2 shRNA sequences. (ii) Proline-rich motif PxxP-SH3 binding site: 2 shRNAs. (iii) Polypurine tract PPT: 3 shRNAs and (iv) Dimerization region: 2 shRNAs.(v) Nef-miRNA: 1 shRNA. (vi) Negative control: 1 shRNA. The functionality of the eleven shRNA sequences was evaluated by measuring the efficiency for inhibition of the *nef-dEGFP* expression from a reporter plasmid after transient cotransfection of Hela T4 cells. The nucleotide sequences of each shRNA are shown en Table 1.

4.3. DESIGN, CONSTRUCTION AND EVALUATION OF GFP BASED REPORTER VECTORS FOR DETERMINATION OF NEF EXPRESSION AND INHIBITION

To assess the effects of shRNAs against *nef in vitro,* the plasmids pd2EGFPN3 and pNef-d2EGFPN3 were constructed as previously described. Both vectors are considered as reporters and the p.Super.retro plasmids expressing shRNAs as effectors. The pd2EGFPN3 is used as one of the positive controls of inhibitory effect of an shRNA specific against GFP. This vector has been previously generated and displays a significant down-regulation efficiency (Figure 10). The pNef-d2EGFPN3 vector was obtained by fusion of the full-length *nef* ORF at the 5' end of *dEGFP* coding sequence using the pEGFPN3 vector, as described in materials and methods (Figure 11).

The functionality of the two reporter vectors was evaluated by transient transfection into HeLaT4 cells. After 48 h of transfection, biochemical analysis, by PAGE and western blot, using a specific rabbit anti GFP monoclonal antibody, showed that both the pd2EGFPN3 and pNef-d2EGFPN3 vectors expressed significant amounts of proteins with molecular weight compatible with dEGFP and Nef-d2EGFPN3. Further characterization of the biological activity of the two proteins was performed by conventional epifluorescece and digital confocal microscopy. Both dEGFP and the fusion protein expression were confirmed in HeLaT4 cells after 48 of

transfection with the two reporter vectors. As expected, the two proteins displayed different patterns of expression and localization.

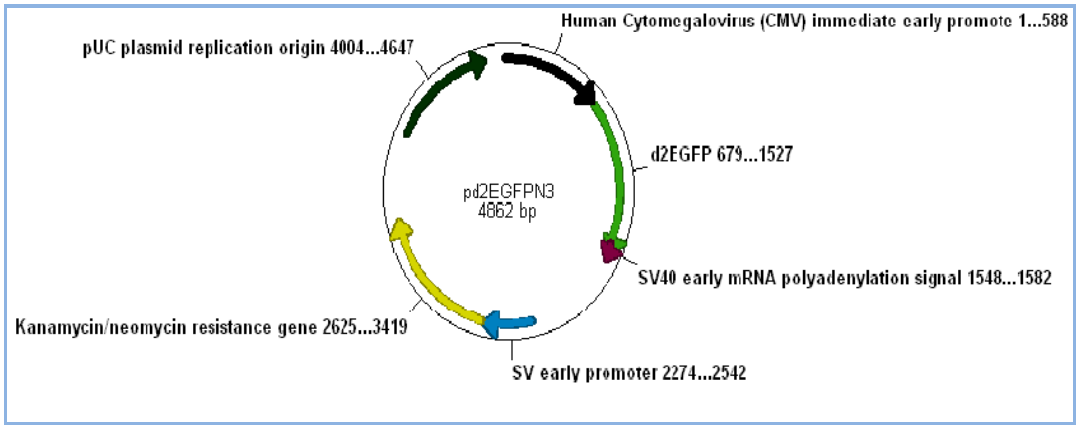


Figure 10. Map of the pd2EGFPN3 vector. The d2EGFP encoding sequence was cloned into the MCS of the commercial vector pEGFP-N3 after removing the sequence of the *EGFP* at the indicated localization. Other general features of conventional expression plasmids are also shown (Davis 2006).

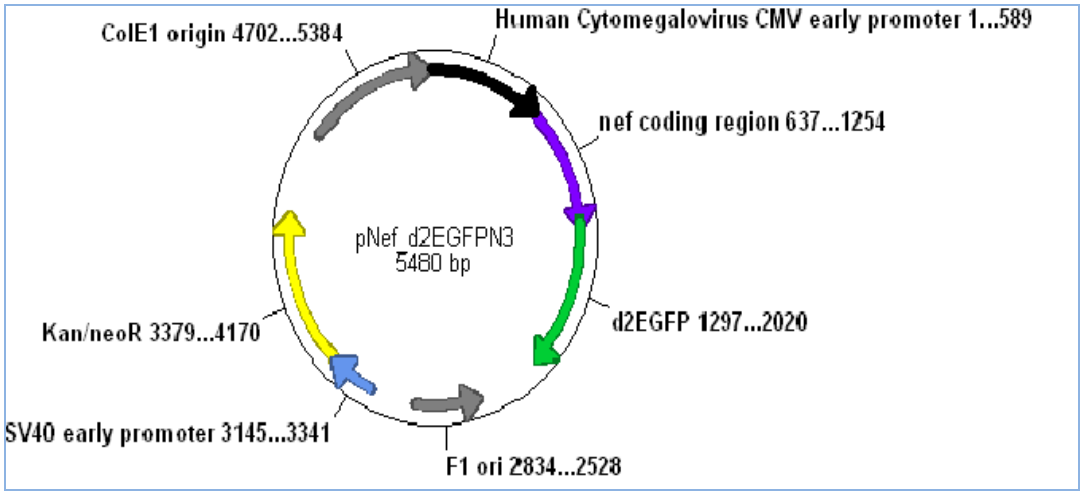


Figure 11 .Map of the pNef-d2EGFPN3 vector.The shows the *nefd2EGFP*sequence cloned into the same backbone of the pEGFP-N3 and other general features of conventional expression plasmids (Davis 2006).

By epifluorescence microscopy the localization of the dEGFP native protein was observed was in the cytoplasm and nucleus (Figure 12 a-b) (Seibel, Eljouni et al. 2007). In contrast, the Nef-dEGFP fusion protein was localized almost at the perinuclear region (Figure 12 c-d). This was in agreement with previous reports, which have demonstrated the 27 kDa Nef was predominantly localized in the cytoplasm of the cell margin of infected cells and associated with membranes (Franchini, Robert-Guroff et al. 1986; Kaminchik, Bashan et al. 1991).

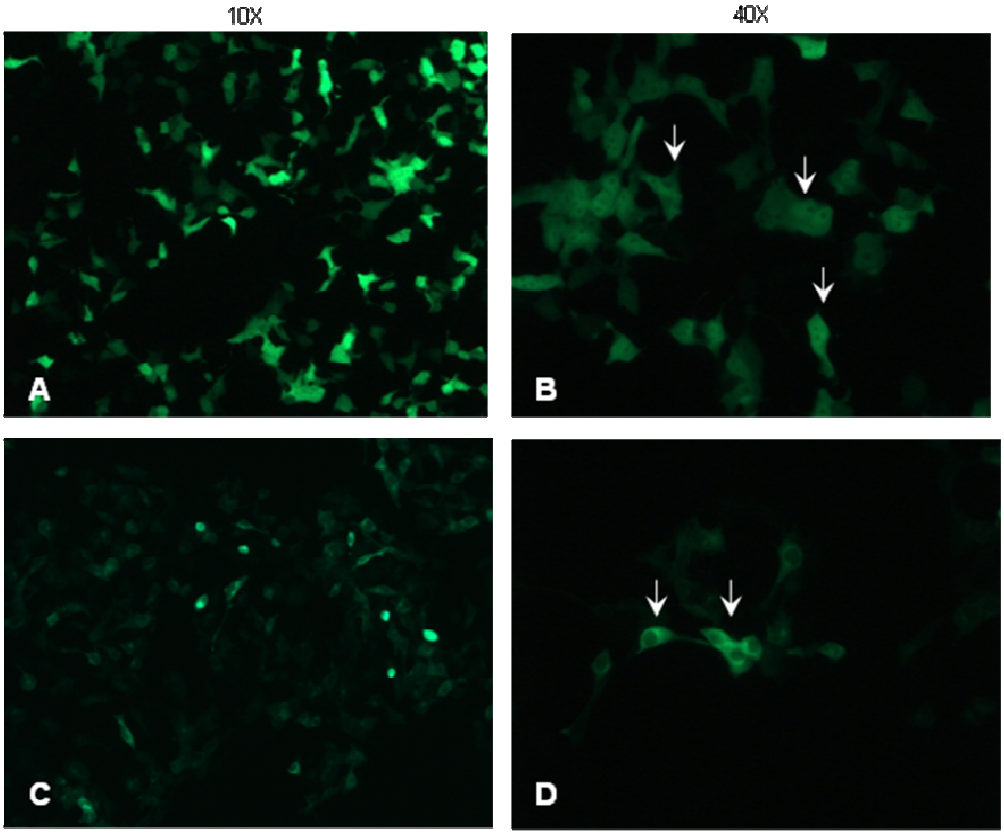


Figure 12. Expression of both dEGFP and Nef-dEGFP reporter proteins. Both expression were first assessed by epifluorescence on live transiently transfected HeLaT4 cells.(A-B) pd2EGFPN3 HeLaT4 cells. (C-D) pNef-d2EGFPN3 HeLaT4 cells.

Remarkably, at the same transfection conditions and efficiency, the fusion protein was expressed at a lower level than the destabilized EGFP native protein.

Additionally, the subcellular localization of fusion protein was confirmed by digital confocal microcopy (Figure 13-b), which showed that indeed the fusion protein was concentrated at perinuclear structures which may correspond to enlarged early/sorting endosomes (Burtey, Rappoport et al. 2007).

Some researchers have suggested toxic effects of Nef in stably transfected murine macrophage cell lines (Murphy, Sweet et al. 1993).

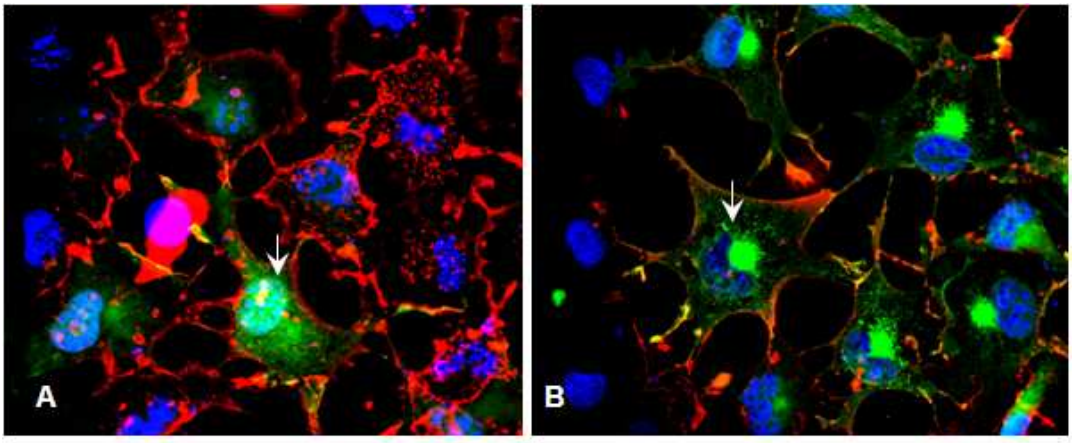


Figure 13. Subcellular localization of dEGFP and fusion reporter protein. Both subcellular localization were assessed by confocal fluorescencefixed transiently transfected HeLaT4 cells. (A) pd2EGFPN3 HeLaT4 cells. (B) pNef-d2EGFPN3 HeLaT4 cells.

4.4 INHIBITION OF THE EXPRESSION OF Nef-dEGFP BY PLASMIDS ENCODING shRNAs IN HeLa T4 CELLS.

The transient co-transfection of these reporter vectors with psuper.retroshRNA expressing vector in HeLaT4 cells should allow observe the RNA interference activity on the mRNA transcripts of *dEGFP* or *nef-dEGFP* after 48 hours, approximately. The Figure 14 shows a schematic representation of the designed experiment and the predicted mechanism and result.

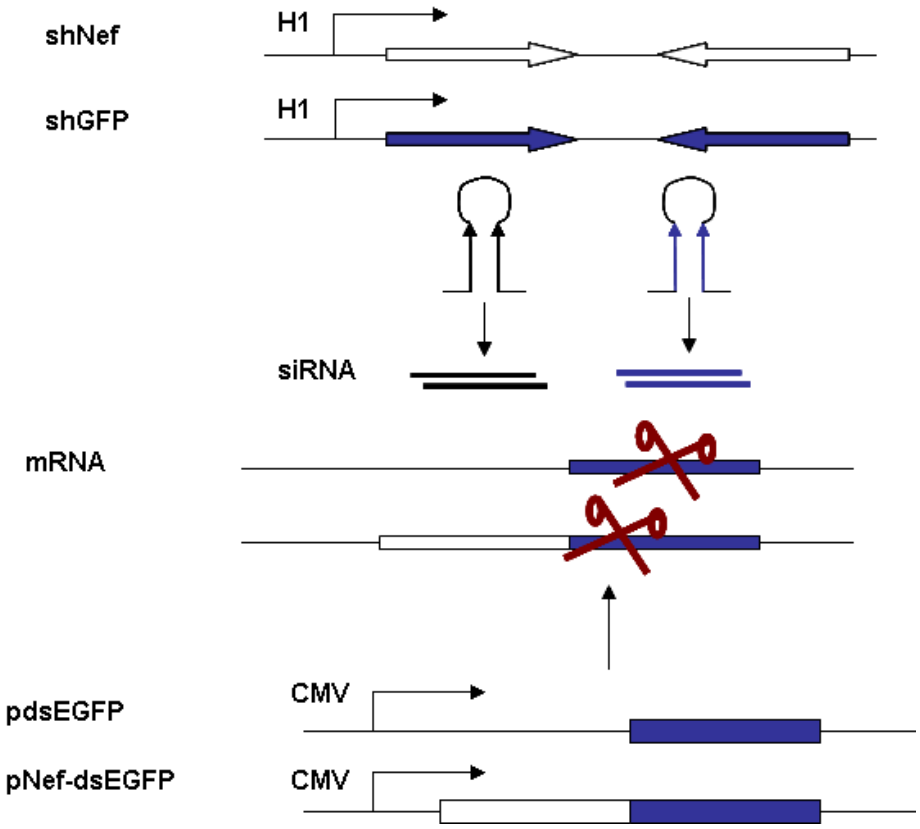


Figure 14. Schematic representation of RNA interference activity from shRNA sequences against EGFP and *nef-dEGFP.* A schematic view of RNA-mediated silencing of siRNA specific sequences on mRNA transcribed from reporter vectors, pd2EGFPN3 and pNef-d2EGFPN3.

To evaluate the level of inhibition on the expression of *Nef-dEGFP* due to the treatment with each of the 11 shRNA sequences, HeLa T4 cells were transfected with 1 µg of fusion reporter vector and 2 µg of every pSRshRNA plasmid. After 48h of transfection, the expression of EGFP was determined, initially, by flow cytometry (figure 15), and later on by other methods.

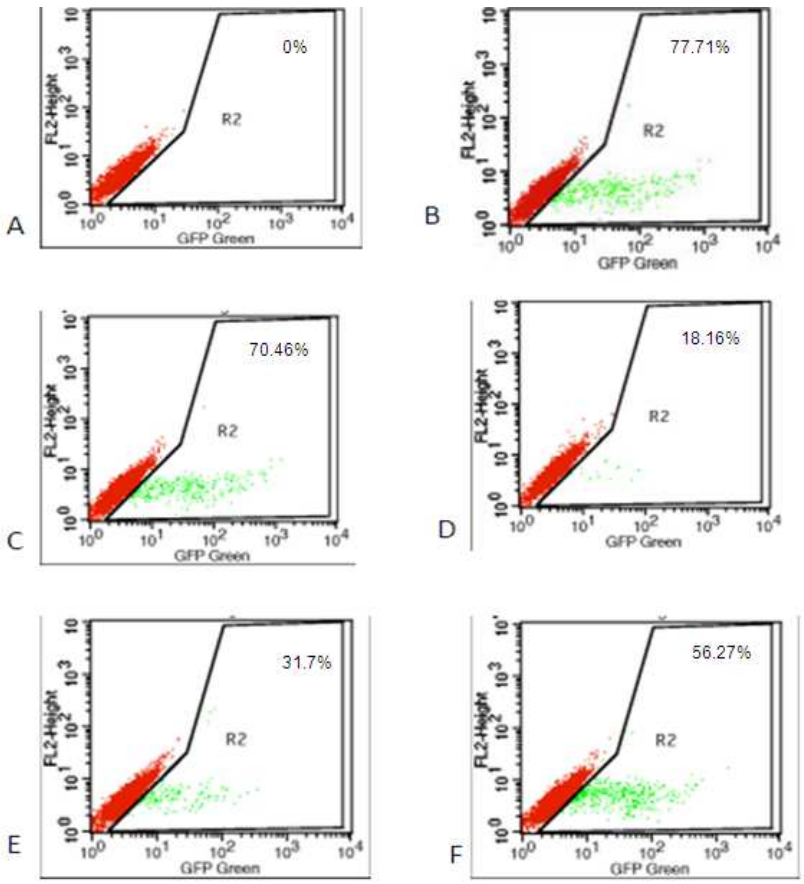


Figure 15. Examples of two-parameter dot plot from the FACS analysis. The dot plot show the number of cells and the percentage of fluorescence level from Nef-GFP (x exe) expressed by HeLaT4 cell after 48 h of co-transfection with one of the vectors expressing specific shRNAs against *nef*. (A) Untransfected HeLaT4 cells. (B) pNef-d2EGFPN3 reporter vector. (C) pNef-d2EGFPN3 co-transfected with an irrelevant shRNA against Rev. (D) Reporter fusion protein treated with shPxxP4. (E) Reporter fusion protein treated with shRNAGFP and (F) Reporter fusion protein vector treated with shNef-miRNA. (X exe green fluorescence intensity; Y exe FL2 channel used to asses auto fluoresce levels. (Green GFP positive cells –all contained in R2; Red: GFP negative cells). The analyses were performed twice.

The number of GFP positive cells (data not shown) and the mean of fluorescence, in arbitrary units, of each cell population was determined (Figure 16) with the

following results: Expression of Nef-EGFP fusion protein was significantly reduced, more than 70% following treatment with: shPxxP4 (18,16±4,02 mean GFP fluorescence intensity) and shPxxP3 (20,50±1,79) compared with the GFP fluorescence intensity measured of the positive control, shRNARev (77,58±10,06). A reduction around 50-60% was showed in: shMyrSig1 (24,29±2,47); shMyrSig2 (29,08±0,04), shRNAGFP (30,73±1,47) and shDimer2 (36±2,98). A RNAi efficiency <50% was considered in: shPPT6 (45,99± 3,5), shNC (45± 3,75), shDimer1 (46,88± 5,81); shPPT7 (53,94± 4,4), shPPT5 (54,3± 0,94) and shNef-miRNA (56,26± 5,7).

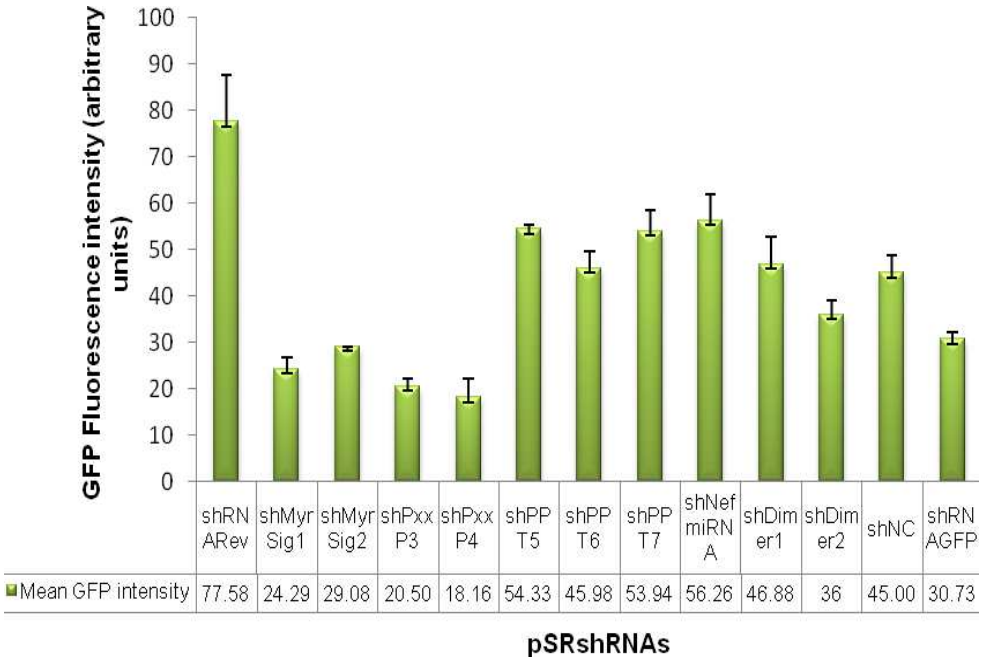


Figure 16. The mean of GFP fluorescence intensity was plotted for each pSRshRNA vector. HeLa cells were transient co-transfected with pNef-d2EGFPN3 and pSRshRNAs. The analyses were performed twice and the standard deviations of both analyses are shown.

Significantly, shRNAs designed against high conserved regions as Proline-rich-PxxP motif-binding SH3 domain Src kinases, which play a key role in Class I

downregulation (Greenberg, Iafrate et al. 1998) and promoting virus replication (Briggs, Scholtz et al. 2001); and the myristoylation region, important for enhancing virus infectivity, CD4 down-modulation, targeting Nef to the cytosolic membrane (Geyer, Munte et al. 1999) and MHC-I negative regulation (Peng and Robert-Guroff 2001) showed a high RNAi efficiency.

However, by this procedure of evaluation, six shRNAs did not show a good level of inhibition. Even, the siRNA designed following the guidelines published and the miRNA encoded by Nef which suppressed Nef expression in vitro (Omoto, Ito et al. 2004). It is worth to mention here that in this particular experiment the efficiency of transfection, probably due to technical fail, was low, and in addition, even a destabilized version of GFP was used, with reduced half life of fluorescence, a single molecule of GFP can give a signal detected by the cytometer. Therefore, FACS analysis with reporter systems of gene expression based on GFP should be considered as a quick and preliminary step of siRNA-activity.

A second approach for a qualitative, but more approximate evaluation of the siRNA-mediated inhibition of expression for a particular gene based on GFP reporter systems is a western blot after PAGE of total proteins obtained from the treated cells. A total protein lysate obtained from HeLaT4 cells after 48h of transfetion, showed in a western blot with an anti GFP monoclonal antibody: first, the expression and the functionality of the two reporter vectors and second, confirmed the significant inhibitory effect on the expression of the dEGFP and fusion proteins by the treatment with a shRNA specific for *GFP* gene. The specificity of this effect was confirmed by treatment with an irrelevant shRNA against *rev* gene, which did not affect the fusion reporter protein expression (Figure 17).

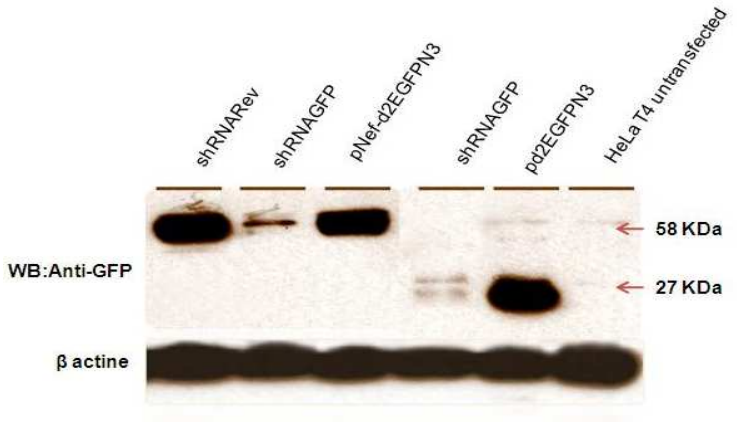


Figure 17. Nef-dEGFP and EGFP expression protein by Western blot. A Western blot was done for the detection of the dEGFP and Nef-dEGFP and evaluation of the functionality of a plasmid expressing a specific anti *nef*shRNA. The GFP protein was detected by a specific anti GFP antibody in a cell lysate obtained from HeLa T4 cells cotransfected with the respective plasmids, after 48 h of transfection. As expected d2EGFP protein present a band of 27 kDa. In contrast, Nef-dEGFP a band of 58 kDa. The inbibition of protein expression by shGFP was significant for both reporter vectors. The co-transfection with shRNARev did not affect the expression. As loading control was used β actin expression.

Next, all the plasmids expressing anti *nef*-specific shRNA were co-transfected into HeLaT4 cells together with the vector expressing the fusion protein. The cells were harvested 48 h post-transfection and a total protein lysate was evaluated in the same way. The highest inhibitory effect was observed in the cells cotransfected with the shRNAs targeting the PxxP motif-SH3 binding to Src kinases, the Dimerization region and the Myristoylation signal. As expected, the shRNA designed by using the SiSearch algorithm (Chalk, Wahlestedt et al. 2004). In addition, the shRNAs against Polypurine region also displayed a substantial inhibitory effect (close to 70%). The shRNA against NefmiRNA showed the lower inhibitory activity (near to 50%). In general, the inhibition of *Nef-dEGFP* expression was substantially higher when it was analyzed by Western blot than by FACS. But, the pattern was conserved (Figure 18).

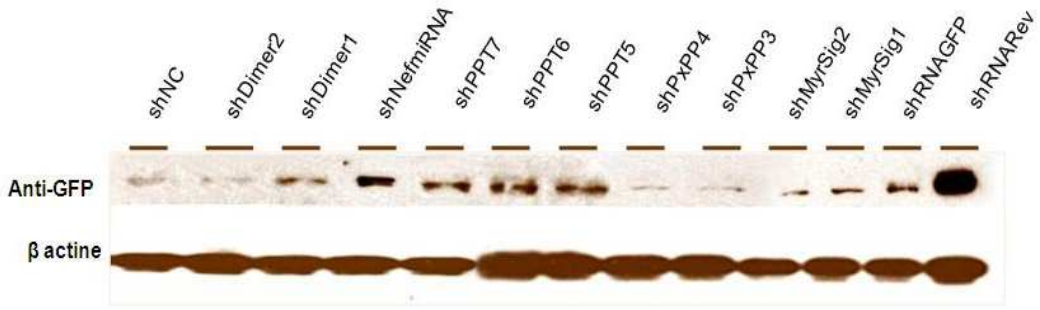


Figure 18. shRNAs targeting *nef* reduced Nef-dEGFP expression. The reduction was visualized by mmunoblotting in total protein extracts from HeLaT4 cells, after 48 h of cotransfection with plasmids expressing anti *nef*-specific shRNAs together with the vector pNef-d2EGFPN3. The fusion protein was detected with an antiserum-GFP. The experiment was performed twice in which an equal apttern was observed. The picture shows the results of one representative experiment. As loading control was used β actin expression. The assays were performed twice.

4.5. shRNAs SUPPRESS *nef-dEGFP* mRNA EXPRESSION

In order to assess the effect of intracellularly-produced specific siRNAs, derived from plasmids coding for appropriate shRNAs sequences, on *nef* mRNA expression, total RNA from HeLa T4 cells was extracted 48h after transient co-transfections with 1µg of reporter vector (pNef-d2EGFPN3) and 2µg of plasmids expressing different sequence-specific shRNA (pSuper.RetroshRNAs) against *nef* gene. Two previously tested vectors coding for specific shRNAs against GFP (pSRshRNA-GFP) and against HIV-1 *rev* (pshRNArev) were used as positive and negative controls of siRNA-mediated postrancriptional inhibition, respectively (Arteaga, Hinkula et al. 2003). Transfections were performed with Polyethyleneimine (PEI).

The integrity of the purified RNA from the two performed methods was assessed by agarose gel electrophoresis, where 18S and 28S intact bands of ribosomal RNA (rRNA) were visualized (Figure 19).

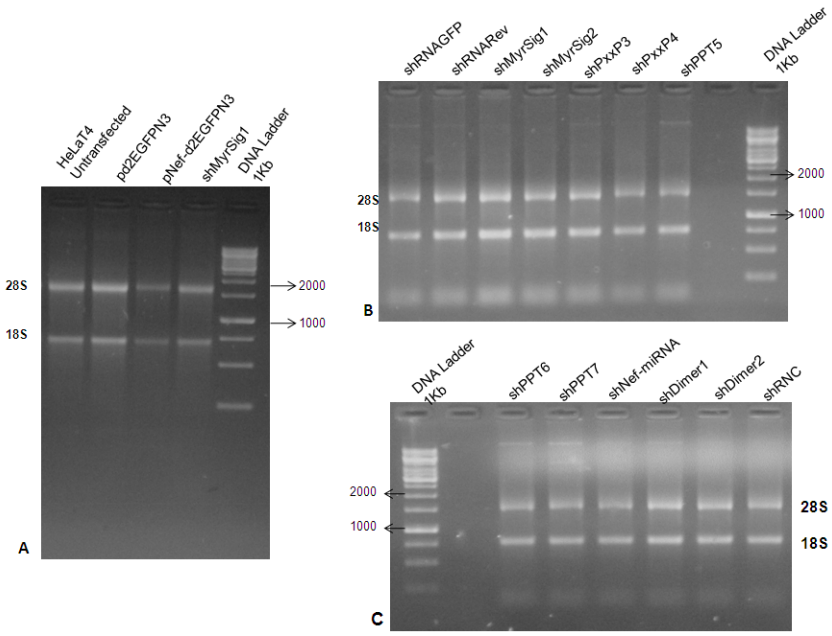


Figure 19. Total RNA isolation. The integrity of the 28s and 18s rRNA bands was visualized by agarose gel electrophoresis of total RNA from HeLa T4 cells, 48h post-cotransfection. A) RNA isolated by SV Total RNA isolation System (Promega). B-C) RNA isolated by TRIzol method (Invitrogen).

Additionally, the yield and quality of total RNA was assessed by determination of the A_{260}/A_{280} ratio by UV spectrophotometry. Total RNA was converted into cDNA and analyzed by qRT-PCR. Each sample was analyzed per triplicate.

Control samples for qRT-PCR with not RNA template (NTC: non-template control) or without reverse transcriptase (RT-: Reverse transcriptase minus) did not show cDNA contamination and/or genomic DNA presence, respectively. Therefore, qRT-PCR for *nef* -the gene of interest (GOI) - and *GAPDH* -a housekeeping gene (HKG) commonly used as control- was performed using RNA samples from HeLa T4 cells treated with each specific anti *nef* or with the shRNA GFP and the shRNA

rev controls, to measure, by relative quantification, the expression levels of the GOI.

RNA from Hela T4 cells cotransfected with the pshRNArev (a plasmid coding for an irrelevant shRNA sequence) was used as a reference and/or untreated sample, for both the expression analysis of the GOI and the HKG. This sample was used because it allows controlling the unspecific inhibition of total protein expression associated with the intracellular presence of foreign genes and unrelated shRNAs.

Regarding the relative quantification analysis of Real Time-PCR data, two methods have been described: (i) the efficiency correction method (Pfaffl 2001) and (ii) the comparative Ct method, also known as the $2^{-\Delta\Delta Ct}$ method(Livak and Schmittgen 2001). Both methods have some advantages. But, they also have some limitations in their assumptions; one of them is about PCR efficiency (*E*) determination and the complexity of the used method to establish it. Although the $2^{-\Delta\Delta Ct}$ model is widely used by most researchers, its principal postulation of target and control efficiencies equaling 2 is not completely fulfilled. For a qRT-PCR with the same amplicon, the efficiencies are different for each individual equal (replicates) and different samples generating individual fluorescence kinetic histories. Even samples with equal RNA input have significant differences on their efficiencies. In addition, qRT-PCR efficiency is influenced by PCR components as reagents, tubes, presence of inhibitors, primers designed, amplicon length, etc. Therefore, in this work has been considered very important to include a mathematical model to correct the efficiency of the reaction for each equal or different sample. Several methods are described in the literature to perform these calculations: (i) the efficiency of the reaction may be determined by running PCR on serial dilutions of the sample under study. When the reactions are completed, the Ct values are determined for each dilution, and are used to obtain log Concentration/Ct plots, then the slope (s) measure is used to establish the Efficiency ($E=10^{1/s}$) (Pfaffl 2001) and (ii) The *E* could be determined also using a linear regression method based on

the exponential segment of the curve and using a standard straight line equation (y=cx+d), where $E=10^c$ (Ramakers, Ruijter et al. 2003).

Notably, Schefe J et al. have described a new efficient and straightforward approach to express relative calculations of a qRT-PCR. This approach called "*rER"* (for *relative expression ratio)* is defined as the calculation of the ratio of the expression levels of a *GOI* with respect to the expression of the *HKG* in a sample-of interest (*SOI)* relative to a reference sample (*ref)* (Schefe, Lehmann et al. 2006). To validate qRT-PCR relative measurement data, this type of analysis considers individual amplification efficiencies for every PCR run on every replicate, sample and gene. To determine the *E* of the PCR a linear regression method is used as described by Ramakers C et al (Ramakers, Ruijter et al. 2003). In brief, this system is based on the linearization of the basic formula for exponential PCR amplification. The log-linear portion of the PCR-progression basic curve can be determined for each sample by selecting a lower and an upper limit of a "window-of-linearity", and the *E* is calculated in this linear range, where a trend line including 3-6 points with the highest possible slope is defined. In addition, an algorithm to estimate the optimal baseline for each individual sample was implemented for this method; all the calculations can be performed by using the LinRegPCR software (11.x version). An alternative data analysis includes the calculation of a mean *E* for each amplicon which may be used to determine the relative expression ratio for each specific gene (Ruijter, Ramakers et al. 2009).

This method (and the software) was used to determine the relative expression of the *nef-dEGFP* fusion gene (GOI) on HeLa T4 cells transiently co-transfected with the pNef-d2EGFPN3 vector and plasmids expressing specific shRNAs against *nef* and also against *GFP* and HIV-1 *rev* as positive and negative control, respectively. Fluorescence background but non-baseline corrected data were exported from the Opticon Monitor 2 software after each PCR run (Figure 20). The program LinRegPCR performed a baseline correction on each sample separately and then a window-of-linearity was determined. Thereafter, a linear regression analysis was

performed to fit a straight line for each PCR throughout the dataset. From the slope of each line the PCR *E* for every individual sample as for each gene as well, was calculated (Figure 21 and 22). As an alternative option, a method considering the mean PCR *E* per amplicon and the individual Ct values were also used. Raw data output are displayed into an Excel spreadsheet.

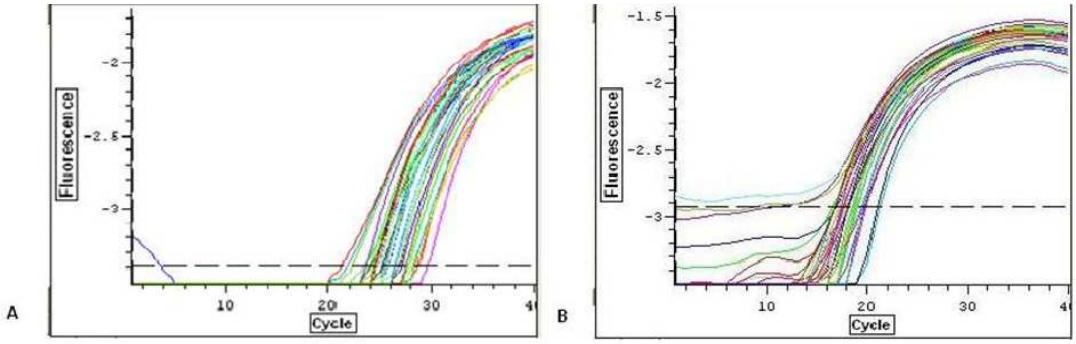


Figure 20. Plot of fluorescence (log scale) against cycle number from qRT-PCR data. Plot displaying background but non-baseline corrected data after qRT-PCR for (A) *nef*-dEGFP and (B) GAPDH. The assays were performed by triplicate.

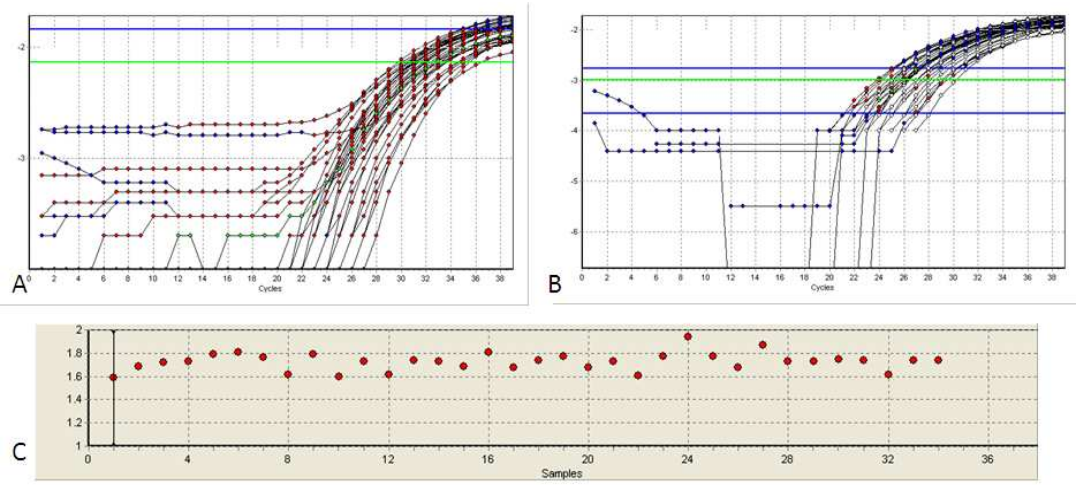


Figure 21. LinRegPCR output graphs of *nef-dEGFP* (GOI) qRT-PCR. The fluorescence levels against cycle number are plotted. (A) Raw data of all the samples immediately after importing a non-baseline corrected values for the GOI set. (B) All the samples were

graphed again after estimation and subtraction of the baseline signal. (C) The individual Es are plotted within a range value from 1.6 to 2 level of fluorescence.

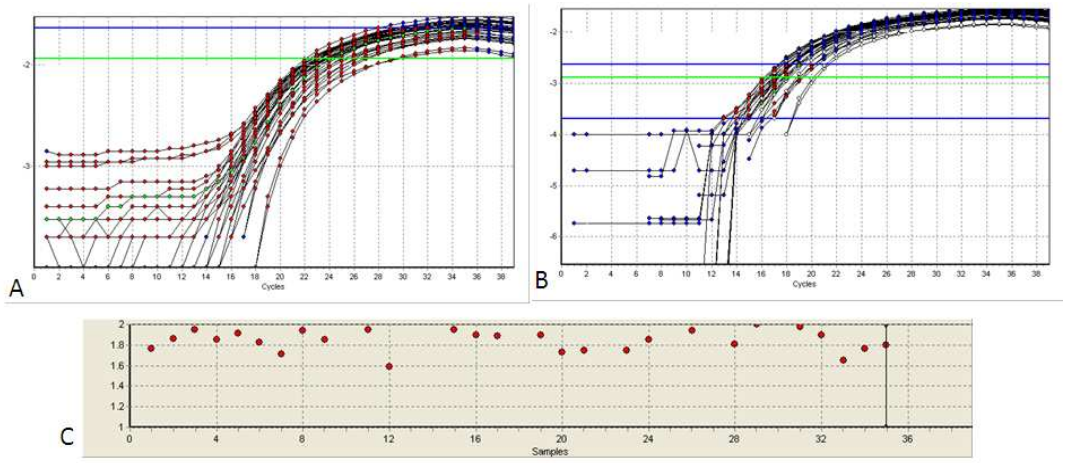


Figure 22. LinRegPCR output graphs of *GAPDH* (HKG) qRT-PCR. The fluorescence levels against cycle number are plotted. (A) Raw data of all the samples immediately after importing a non-baseline corrected values for the HKG set. (B) All the samples were graphed again after estimation and subtraction of the baseline signal. (C) The individual Es are plotted within a range value from 1.6 to 2 level of fluorescence.

For determination of the expression level of the GOI and the HKG, each sample was tested by duplicate or triplicate. The *E* and the Ct per well of individual samples were calculated and averages and standard deviation (SD) of the replicates were determined. The individually averaged *E* and Cts for each gene qRT-PCR in every sample are shown in Table 3. The mean *E* and the Ct obtained from a sample of the same type of cells treated with a plasmid expressing a shRNA sequence against the HIV-1*rev* (unrelated shRNA) were used as reference values.

The level of expression of the *nef-dEGFP* were measured as the *rER*, calculated first applying a formula (Formula 1) that considers the mean of *E* and Ct values both for the GOI (*nef-GFP*) and the HKG (*GAPDH*) for each individual sample, using the LinRegPCR program.

$$rER = \frac{Rnorm(SOI)}{Rnorm(ref)} = \frac{\left(1 + E(HKG;SOI)\right)^{Ct(HKG;SOI)}}{(1 + E(GOI;SOI)^{Ct(GOI;SOI)}} \times \frac{(1 + E(HKG;ref))^{-Ct(HKG;ref)}}{(1 + E(GOI;ref))^{-Ct(GOI;ref)}} \quad 1$$

Alternatively, the mean *E* for each amplicon (PCR for *nef-GFP* or *GAPDH*) was used to establish the *rER* for the same gene with an alternative formula that uses in addition the ΔCt between the treated or problem sample (specific shRNAs against nef) and the reference sample (in this case, shRNA*rev* treated sample) (Formula 2).

$$rER = \frac{Rnorm(SOI)}{Rnorm(ref)} = \frac{(1+E(GOI))^{-\Delta Ct(GOI)}}{(1+E(HKG))^{-\Delta Ct(HKG)}} \quad 2$$

The mean *E* for the *nef* qRT-PCR was 1,708 and for the *GAPDH* was 1,861. (Two samples pNef-dEGFPN3+pSRshRNAMyr_1 and pNef-d2EGFPN3+pSRshNef-miRNA were not evaluated due to sample lost).

In general, the expression level of *nef-GFP,* relative to the *GAPDH,* in samples treated with shRNAs against *nef* with reference to the expression in the sample treated with an unrelated shRNA (against HIV-1 *rev*), which is considered the 100% level of expression, showed a high efficiency of inhibition in most of the samples above 90% (Figure 23).

Calculations using the individual mean of *E* and Cts for each sample in each gene revealed, as expected, a very high level of inhibition of the *nef-dEGFP* expression by shRNAs against *GFP*, 1.33 % of mRNA expression.

Table 3. Average and SD of Es and Cts from triplicate of *nef-dEGFP* and *GAPDH* qRT-PCR from individual samples

	***nef* data set**				**GAPDH data set**			
Samples	Mean E	DS E	Mean Ct	DS Ct	Mean E	DS E	Mean Ct	Ds Ct
shRNARev	1,70	0,089	24,97	0,22	1,79	0,047	17,74	0,15
shRNAGFP	1,77	0,030	29,84	0,49	2,11	0,043	18,06	0,57
shMyrSig2	1,67	0,071	26,18	0,19	1,87	0,030	15,86	0,05
shPxxP3	1,77	0,050	25,60	0,61	1,85	0,047	16,18	0,20
shPxxP4	1,82	0,070	27,22	0,29	1,95	0,004	16,74	0,34
shPPT5	1,69	0,073	26,13	0,26	1,93	0,178	16,19	0,09
shPPT6	1,70	0,031	25,53	0,40	1,94	0,009	17,17	0,20
shPPT7	1,74	0,020	25,10	1,09	1,82	0,102	16,72	0,15
shDimer1	´1,77	0,050	28,50	0,82	1,85	1,56E-05	16,90	1,86E-05
shDimer2	1,73	0,004	29,18	0,10	1,99	0,14	18,92	0,02
shNC	1,73	0,058	27,52	0,55	1,94	0,04	18,18	0,76

A robust inhibition efficiency close to 95% was observed in samples treated with shRNAs against: (i) Dimerization region: shDimer1 (1,4%) and shDimer2 (5,84%), a region implicated in the formation of oligomers, leading to a more efficient interaction of Nef with its cellular partners, such as Hck kinases, at the plasma membrane (Ye, Choi et al. 2004) ; (ii) Proline-rich (PxxP) motif: shPxxP4 (4,32%), this motif shows specific binding to SH3 domains of Src Family kinases, like Hck and Lyn, promoting its tyrosine kinase activity at T cell activation pathway (Moarefi, LaFevre-Bernt et al. 1997); (iii) Myristoylation signal region: shMyrSig2(5,38%), a region of acidic charge, enabling Nef to localize at the plasma membrane and interact with adapter proteins of the endocytic machinery (Geyer, Munte et al. 1999). Notably, these regions are highly conserved (Shugars, Smith et al. 1993).

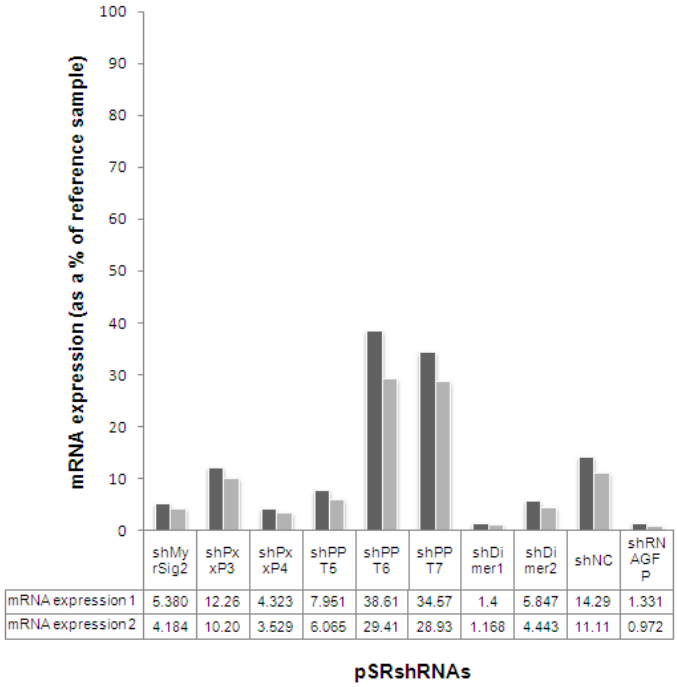


Figure 23. Expression level of mRNA *nef-dEGFP*. Dark grey bars represent the mRNA expression level using mean of individual E and Ct for each sample in each gene. Light grey bars represent the mRNA expression using the mean E for each gene and a ∆ Ct for each sample.

In addition, good RNAi efficiencies of more than 85% were observed in samples from: (i) 3' Polypurine tract: shPPT5 (7,95%), an essential region for the precise plus-strand initiation in the reverse transcription (Rausch and Le Grice 2004); (ii) Proline-rich (PxxP) motif : shPxxP3 (12,26%); (iii) Negative control: shNC (14,29%). Interestingly, this region is not highly conserved. But, the siRNA sequence was designed according basic guidelines published and included in siSearch software tool (Chalk, Wahlestedt et al. 2004). Finally, significant efficiencies close to 70% were observed in samples from Polypurine region: shPPT6 (38,61%) and shPPT7 (34,57%). Although, the target sequence is conserved, these shRNAs contain more Gs stretches than shPPT5, probably enabling a secondary RNA structure, which could block the RNA cleavage at RISC.

Calculations using the mean E for each gene showed a slight reduction of mRNA expression for all shRNAs compared with the individual mean E for each sample. Indeed, these data revealed a high RNAi efficiency among the plasmid encoding shRNAs against conserved and strategic target regions of the HIV-1 *nef* , involved in essential roles such the modulation of signaling pathways in T cell activation and down-regulation of CD4 receptor, which together enhance the viral infectivity.

The melting curve obtained from the qRT-PCR of each gene did not show any primer dimmers, contaminating DNA, and PCR product from mis-annealed primer. The inflection point or melting temperature of the amplicon corresponding to *nef* and *GAPDH* product was 79 and 80℃, respectively (Figure 24 A and B).

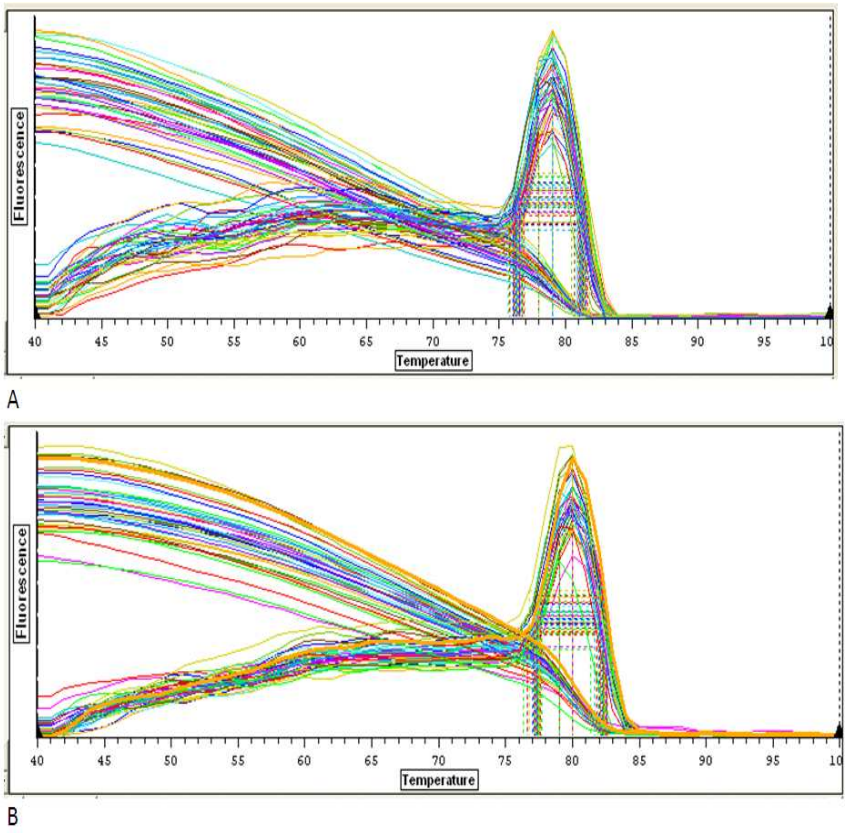
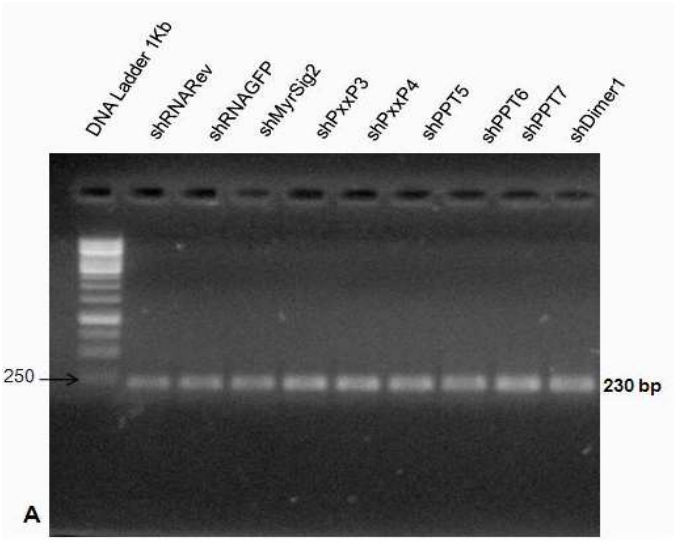
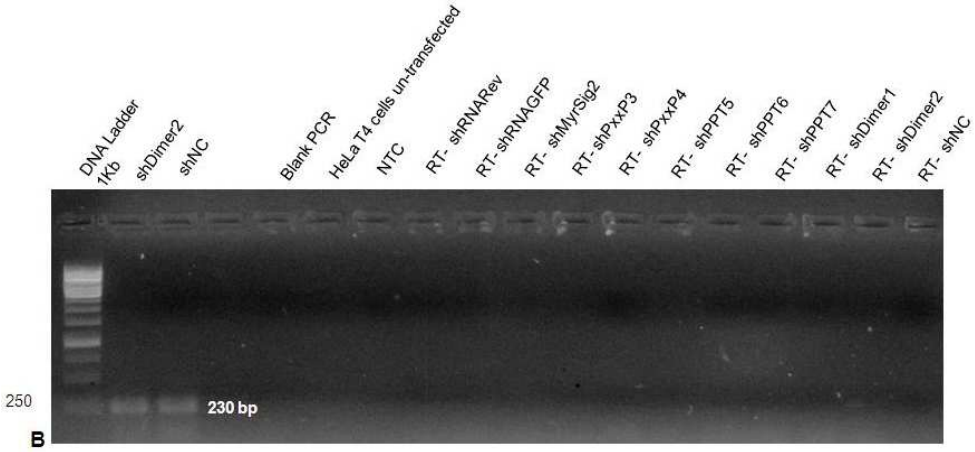


Figure 24. Melting curves. Fluorescence vs. temperature plots were performed after PCR cycles for (A) *nef* and (B) *GAPDH.*

The amplification products were additionally visualized after electrophoresis on agarose gel to verify the specific product of *nef* (230 bp) in samples cotransfected with reporter vector and treated with shRNAs (Figure 25A). Any un-specific product was visualized on cDNA synthesis controls: NTC and RT- (Figure 25B). Likewise, the specific product of *GAPDH* (113 bp) was assessed (Figure 25 C).





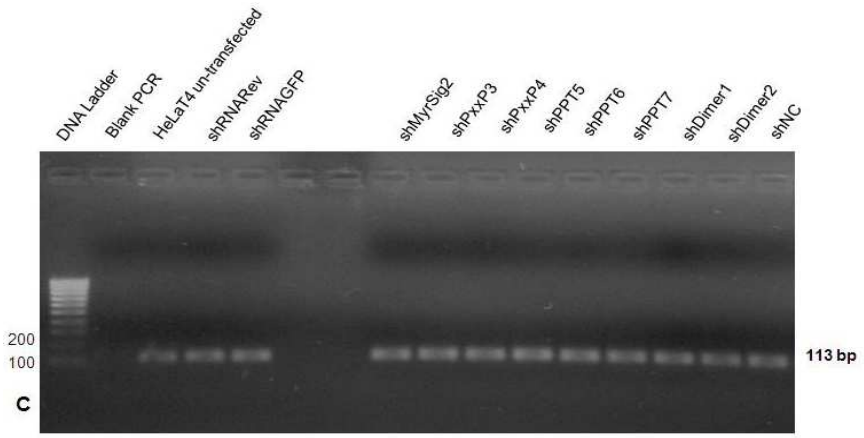


Figure 25. Agarose gels electrophoresis of qRT-PCR amplification products. (A, B) *nef* qRT-PCR amplicons. The positive samples, a band of 230 bp, and cDNA controls were run on an agarose gel. C) GAPDH qRT-PCR products (113 bp).

4.6 EVALUATION OF shRNAs DESIGNED AND COMPARISON WITH THEIR in *vitro* PERFORMANCE

The design of the shRNAs sequences against *nef* in this study was aimed to target highly conserved regions coding for protein domains playing key roles on HIV-1 infectivity. However, it has been demonstrated that finding good siRNAs is a difficult work. Even more, given that the *nef* target regions, selected under the above-mentioned criteria, restrict the possibilities of always finding sequences that fit the rules for selection of siRNA targets from the different available algorithms, we designed the shRNAs used in this study, by hand.

As an exercise for future works, an evaluation of the shRNAs tested in this work for a predicted efficiency based on pre-established rules and the results from the experimental data was carried out.

Several factors, such as cellular, molecular and sequence based features may influence the inhibition efficiency of siRNAs (Pancoska, Moravek et al. 2004).

Therefore, basic rules have been established to design siRNAs molecules having high probability of good functionality (Elbashir, Harborth et al. 2002). Additionally, considering new issues such as thermodynamic and structural constraints could improve the efficiency of these molecules. This new features have been included on different rules and algorithms greatly accepted by reserachers working on siRNA; two of them are: (i) the Ui-Tei rules (Ui-Tei, Naito et al. 2004) and (ii) the Reynolds guidelines (Reynolds, Leake et al. 2004).

To evaluate the shRNA*nef* sequences designed by hand, according to the two mentioned guidelines, every shRNA was evaluated for each parameter and a class scoring was established as proposed for the authors of each algorithm. The table 4 describes the parameters according Ui-Tei et al (Ui-Tei, Naito et al. 2004) and the table 5 listed the shRNAs and their description based on Reynolds (Reynolds, Leake et al. 2004). For comparison the shNC, the only sequence designed by an algorithm, was also evaluated.

Table 4. Evaluation of shRNAs against *nef* according to Ui-Tei K et al parameters (Ui-Tei, Naito et al. 2004).

shRNA*nef* designed	A/U at the 5' end of antisense strand (AS)	G/C at the 5' end of the sense strand	At least five A/U residues in the 5' terminal one-third of the AS	The absence of any GC stretch of more than 9 nt in length	Class
shMyrSig1	+	-	3 U and 1 A	+	II
shMyrSig2	+	-	2 U and 1 A	+	II
shPxxP3	+	+	2 A and 1 U	+	II
shPxxP4	+	+	3 A and 1 U	+	I
shPPT5	+	+	2 U and 3 A	+	I
shPPT6	+	+	1 A and 2 U	+	II

shPPT7	-	-	2 U	+	III
shNef-miRNA	+	+	2 A	+	II
shDimer1	-	+	2 U and 1 A	+	II
shDimer2	+	+	1 A and 2 U	+	II
shNC	+	+	3 U	+	II

+: Fulfill the criteria; **-**: Do not fulfill the criteria.

Table 5. Evaluation of shRNAs against *nef* according Reynolds parameters.

shRNA targeting *nef*	Criterion I. 30-52% G/C content	Criterion II. Three or more A/U in positions 15-19 of sense strand (ss)	Criterion III. Tm <20℃- lack of internal repeats	Criterion IV. An A at position 19 ss	Criterion V. An A at position 3 ss	Criterion VI. An U at position 10 of ss	Criterion VII. Absence of a G or C at position 19 of ss	Criterion VIII. Absence of a G at position 13 of ss	Score
shMyrSig1	52,6	1 U and 3A	46℃	+	-	-	+	-	5
shMyrSig2	52,6	1 U and 2A	51,1℃	+	-	-	+	+	6
shPxxP3	52,6	1 A	46℃	-	-	-	+	-	1
shPxxP4	43,47	3 U and 1A	44℃	+	-	-	+	+	7
shPPT5	52,63	3 U and 2A	46℃	-	-	-	+	-	5
shPPT6	63,16	1 U and 2A	50℃	+	-	-	+	-	4
shPPT7	52,63	2 A	46℃	-	+	-	-	-	1
shNef-miRNA	52,6	2 U	55℃	-	-	-	+	+	4
shDimer1	42	1 U and 2A	48,9℃	-	-	-	-	-	1
shDimer2	47,7	1 U and 2A	44℃	+	-	-	+	+	6
shNC	52,63	3A	51,1℃	+	+	-	+	+	7

+: Fulfill the criteria. -: Do not fulfill the criteria. A score of 6 or more in any siRNA is considered as a predictive approach for functional sequences.

Based on the previous description and according Ui-Tei guidelines (Table 4), two shRNAs, the shPxxP4 and the shPPT5, were classified as class I (highRNAi efficiency).

If this analysis is compared with the qRT-PCR results, shPxxP4 and, to a slight lower extent, shPPT5 (class I) with inhibition efficiencies of more than 95 and 92% respectively, were verified as a highly efficient shRNAs against *nef.* Notably, the rest of shRNA efficiencies, classified as class II, coincide, in some extent, with the evaluation by Ui-Tei rules, except shPPT6, which in the qRT-PCR experiments showed efficiency between 38.61 and 29.41 %, being the less efficient shRNA. The shPPT7 sequence was classified as a class III shRNA and indeed was one of the less effective vectors (34.57–28.93 % of efficiency). The rest of the vectors showed efficacies in most of the cases close or higher than 90%. These according to some authors may be considered a high level of efficiency.

Classifying the shRNAs according to the Reynolds guidelines (Table 5), 4 shRNAs (shPxxP4, shNC, shMyrSig2 and shDimer2) had a score higher than 6, whit predicted inhibitory efficiency of more than 80%. This was in clear agreement with the experimental results. On the other hand, 5 shRNAs: shMyrSig1, shPPT5, shPxxP3, shPPT7, shDimer1- got a score lower than 5 and therefore were predicted to be nonfunctional siRNAs, with inhibition lesser than 50%. Except shPPT7, which has one of the lowest efficiencies (34.57 – 28.93 %), all of the other shRNAs have efficiencies higher than80%. Even more, shMyrSig1 and shPxxP3, which in the qRT-PCR experiments showed a good efficiency, were scored as nonfunctional by Reynolds rules.

These comparisons suggest that in case of some technical or strategic constrains for designing shRNAs according to the most common used rules proposed for different authors, a careful design by hand using the rules just as guidelines could be an efficient strategy for the design of reasonably good enough efficient shRNAs.

Recently, Taxman and colleagues showed that the Reynolds guideline do not provide positive correlative criteria for shRNA design. In this work, the algorithm of Ui-Tei correlated better than the one of Reynolds, with shRNA efficacy, although perhaps some modifications concerning thermodynamic features are still required to get stronger correlations with the experimental data (Taxman, Livingstone et al. 2006).

5 DISCUSSION

HIV-1 is one of the infections in which the knowledge of the viral genes, proteins and the essential mechanisms involved in cellular processes and interactions between the virus and the host cells has been most extensively described. This knowledge has allowed designing and developing different antiviral methods, leading to important therapeutic advances.

Since RNAi discovery, several approaches have been performed, such as synthetic siRNAs or shRNAs sequences targeting different HIV-1 genes. A remarkable inhibitory activity of viral replication by this method was reported at the very early stages of the development of the technology (Park, Miyano-Kurosaki et al. 2002; Arteaga, Hinkula et al. 2003; Das, Brummelkamp et al. 2004; Chang, Liu et al. 2005).

Nef protein even though is an accessory protein and was considered as a repressor of HIV-1 replication in earliest studies of HIV-1 infection, is currently regarded as a very promising target for siRNA silencing. An extensive record of Nef interactions with several cellular proteins, affecting different functions has been described until now and most of them are referenced in the HIV-1, Human protein interaction database (NCBI). Although the implications of several of these interactions are still unclear, important functions have been described. Briefly, Nef affects cells in many ways: down-regulating CD4 receptor and MHC class I expression and altering T-cell activation and maturation, among others. Additionally, *nef* sequences deleted or mutated have been found in LTNP, suggesting a direct link between *nef* and viral pathogenesis and infectivity. Likewise any other HIV-1 genes targeted by siRNA, such as *tat*, *rev* and *gag-pol,*

different assays targeting *nef* have shown successful inhibition of HIV-1 infectivity and replication.

One of the hallmarks of RNAi, is sequence specificity. However for some purposes, such as antiviral therapy, this is also one of the main drawbacks because a single nucleotide substitution in the target region is enough to abolish completely the inhibitory activity. In addition, one of the main HIV-1 features is the high mutation rate on the reverse transcription process. As a consequence, when using siRNA for inhibiting HIV-1 replication, a quick emergency of viral escape mutants is almost always observed. Therefore, it has been suggested different mechanisms to avoid this negative aspect, siRNAs against HIV-1 should target highly conserved sequences of the viral genome (Chang, Liu et al. 2005). However, most of the effective siRNAs against HIV-1 targets, which has been published until now, are in fact no highly conserved. Interestingly, it is observed, as described here that Nef shows at least four sequences of residues highly conserved, which constitute functional motifs playing key roles on HIV-1 infectivity (Shugars, Smith et al. 1993). In addition, two of these motifs have a high structural complexity which, probably are essential for a proper functionality.

For an effective siRNA based gene therapy against HIV-1 infection, which avoids in great extent the generation of viral siRNA escape mutants, an approach has been suggested in this work. The established criteria to design siRNA sequences were mainly: Targets with highly conserved sequences and endowed with essential functional activities of Nef. In addition motifs with these two feature, plus a complex structure, which most probably is needed for functional protein-protein interactions could be the best targets. The hypothesis behind these reasoning is that non-silent mutations at these sites could cause lost of function of the protein or select replicative but very low virulent strains of the virus. In any case if escape mutants arise by selection of strains with silent mutations one more option is the combination of more than one siRNA against this type of strategic targets. Finally,

targets encoding for more than one ORF could be one more strategy to overcome the problem of silent mutations.

As a device to search the sequences, the HIV sequence alignments tool on HIV sequence Database from Los Alamos National laboratory was available. This instrument was used in order to analyze highly conserved sequences on both nucleotide and protein level (Leitner 2006) and identify the motifs on Nef sequence that fulfill in some extend the features for strategic siRNA targets against HIV-1 proposed in this thesis (Figure 8). Therefore, based on the alignment with the *nef* gene and protein sequences from consensus/ancentral HIV-1 group and using the available literature, four siRNA target sequences were chosen:

(i) Myristoylation signal: mediates a post-translational modification which directs Nef to the cell membrane (Kaminchik, Bashan et al. 1991). Also, a myristoylated Nef protein is able to associate with the T cell cytoskeletal matrix (Niederman, Hastings et al. 1993), down-regulate CD4 and MHC-I expression and interacts with molecules involved in the endocytosis pathway (Arold and Baur 2001). This is a highly conserved sequence, associated with essential functions but with a linear structure.

(ii) Proline-rich type II motif (PxxP) SH3-binding Proteins: the interaction of Nef(PxxP) with two Src kinases, Hck and Lyn kinases, trigger T cell activation pathway (Moarefi, LaFevre-Bernt et al. 1997). In addition, it promotes virus replication (Briggs, Scholtz et al. 2001). This highly conserved region in addition of playing a number of key functions for viral replication, has also a very high structural complexity.

(iii) Polypurine tract (PPT): It functions as the plus strain RNA primer in reverse transcription (Pullen, Rattray et al. 1993). This target is essential for viral replication. Additionally, it is important for RNA-RNA interactions, has in some extent structural complexity and is a conserved sequence. This region will not

suffer the limitation of silent mutations because of its primer function and because in addition this region involves also segments that constitute the 3´LTR

(iv) Dimerization region: mediates Nef establishment of dimeric, trimeric and tetrameric polymerization arrangements (Kienzle, Freund et al. 1993). This permits to create a better contact with cellular proteins involved in signaling or endocytic pathways (Arold, Hoh et al. 2000).This is a domain with almost linear structure, but the conserved sequence is an indication of the importance of the motif for viral replication.

In addition, two additional RNAi duplex were designed. First, a negative control for the criteria established, was chosen by an algorithm namely siSearch, which include the guidelines for shRNA design, such Ui-Tei, Reynolds and Stockholm rules (Chalk, Wahlestedt et al. 2004). Therefore, this shRNA sequence is not highly conserved and it does not implicated in functional interactions. It is expected that in experiments of HIV-1 infection, escape mutants will arise sooner than in the selected strategic sequences. Second, the sequence of a miRNA encoded by HIV-1 was used as a positive control (Omoto, Ito et al. 2004), in order to compare the RNAi efficiency obtained as miRNA vs siRNA sequence.

So far, the studies published regarding the inhibition of HIV-1 replication targeting *nef* gene have not employed similar approaches. Markedly, siRNA duplexes against *nef*, approximately at 54-60 aa region, showed a significant inhibition in HIV-1 genome (Jacque, Triques et al. 2002). However, Das and colleagues reported a resistance to RNAi, mainly due to a deletion of 106 bp. Also, one nucleotide substitution with a further mutation was reported after 62 days (Das, Brummelkamp et al. 2004). In addition, Dave et al reported a ssiRNA targeting the PPT region exerting strong antiviral effects on some neurotropic strains of HIV-1 and on strain NL4-3 (Dave and Pomerantz 2004). Nevertheless, it did not show good RNAi efficiency in a further analysis. Recently, Yamamoto et al focused on

the U3 region overlapped with *nef* and by a web-based program designed a shRNA directed to dimerization region. The transfection of this shRNA reduced *nef* mRNA expression over 90% (Yamamoto, Miyoshi et al. 2006).

In spite of the constrains that are associated with the selection of siRNA targets against the HIV-1 genome fulfilling the requirements established in this work, a careful selection by hand of the most appropriated motifs, allowed in this work to generate shRNA with high inhibitory efficiency against four strategic regions. These target sequences worked efficiently in spite that the algorithms and most accepted rules for the design of siRNAs predicted the contrary.

We proposed these new generated molecules as tools with an improved design as the previously reported until now and with a potential value for the gene therapy of HIV-1 infection. However a closer view of its performance, efficiency and improvement for overcoming, the main limitation of siRNA against HIV-1, that is, the selection of viral escape mutants must be obtained in experiments of long-term infection using cell lines stably expressing this shRNAs. These types of experiments are currently going on.

Previous studies, have suggested that the application of multiple shRNAs simultaneously, preferably targeting highly conserved HIV-1 regions are most effective for the inhibition of HIV-1 replication with siRNA (Berkhout 2004). We believe that in this work several highly functional shRNA sequences against an important segment of the HIV-1 genome, the one coding for the Nef protein, has bee generated. In addition to fitting the requirement of targeting conserved regions they also have additional potential advantages i. e, they target regions, with essential functions of the protein, some of this functions in addition to the sequence the depend on the structure and others are probably les susceptible to be inefficient by the appearance of silent mutations. Modifications of cell lines, including two or more of the shRNAs against nef with the highest inhibitory effect

found in these preliminary studies could be suitable for future in vitro model experiments of HIV-1 infection aimed to evaluate the appearance rate of HIV-1 siRNA resistant strains.

6 REFERENCES

Ahmad, N. and S. Venkatesan (1988). "Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR." Science **241**(4872): 1481-5.

Aiken, C., J. Konner, et al. (1994). "Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain." Cell **76**(5): 853-64.

Akari, H., S. Arold, et al. (2000). "Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation." J Virol **74**(6): 2907-12.

Akashi, H., M. Miyagishi, et al. (2005). "Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA." Mol Biosyst **1**(5-6): 382-90.

Ambros, V., R. C. Lee, et al. (2003). "MicroRNAs and other tiny endogenous RNAs in C. elegans." Curr Biol **13**(10): 807-18.

Arganaraz, E. R., M. Schindler, et al. (2003). "Enhanced CD4 down-modulation by late stage HIV-1 nef alleles is associated with increased Env incorporation and viral replication." J Biol Chem **278**(36): 33912-9.

Arold, S., F. Hoh, et al. (2000). "Characterization and molecular basis of the oligomeric structure of HIV-1 nef protein." Protein Sci **9**(6): 1137-48.

Arold, S. T. and A. S. Baur (2001). "Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein." Trends Biochem Sci **26**(6): 356-63.

Arteaga, H. J., J. Hinkula, et al. (2003). "Choosing CCR5 or Rev siRNA in HIV-1." Nat Biotechnol **21**(3): 230-1.

Arya, S. K. (1987). "3'-orf and sor genes of human immunodeficiency virus: in vitro transcription-translation and immunoreactive domains." Proc Natl Acad Sci U S A **84**(15): 5429-33.

Arya, S. K. and R. C. Gallo (1986). "Three novel genes of human T-lymphotropic virus type III: immune reactivity of their products with sera from acquired immune deficiency syndrome patients." Proc Natl Acad Sci U S A **83**(7): 2209-13.

Atkins, K. M., L. Thomas, et al. (2008). "HIV-1 Nef binds PACS-2 to assemble a multikinase cascade that triggers major histocompatibility complex class I (MHC-I) down-regulation: analysis using short interfering RNA and knock-out mice." J Biol Chem **283**(17): 11772-84.

Azorsa, D. M., S; Caplen, NJ (2003). "Gene silencing through RNA interference: Potential for therapeutics and functional genomics." Letters in Peptide Science **10**(3-4): 361-372.

Baur, A. S., G. Sass, et al. (1997). "The N-terminus of Nef from HIV-1/SIV associates with a protein complex containing Lck and a serine kinase." Immunity **6**(3): 283-91.

Bennasser, Y. and K. T. Jeang (2006). "HIV-1 Tat interaction with Dicer: requirement for RNA." Retrovirology **3**: 95.

Bennasser, Y., S. Y. Le, et al. (2005). "Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing." Immunity **22**(5): 607-19.

Bennasser, Y., S. Y. Le, et al. (2004). "HIV-1 encoded candidate micro-RNAs and their cellular targets." Retrovirology **1**: 43.

Bennasser, Y., M. L. Yeung, et al. (2006). "HIV-1 TAR RNA subverts RNA interference in transfected cells through sequestration of TAR RNA-binding protein, TRBP." J Biol Chem **281**(38): 27674-8.

Berger, E. A., P. M. Murphy, et al. (1999). "Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease." Annu Rev Immunol **17**: 657-700.

Berkhout, B. (2004). "RNA interference as an antiviral approach: targeting HIV-1." Curr Opin Mol Ther **6**(2): 141-5.

Bernstein, E., A. A. Caudy, et al. (2001). "Role for a bidentate ribonuclease in the initiation step of RNA interference." Nature **409**(6818): 363-6.

Boden, D., O. Pusch, et al. (2003). "Human immunodeficiency virus type 1 escape from RNA interference." J Virol **77**(21): 11531-5.

Boden, D., O. Pusch, et al. (2004). "Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins." Nucleic Acids Res **32**(3): 1154-8.

Bresnahan, P. A., W. Yonemoto, et al. (1998). "A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor." Curr Biol **8**(22): 1235-8.

Briggs, S. D., B. Scholtz, et al. (2001). "HIV-1 Nef promotes survival of myeloid cells by a Stat3-dependent pathway." J Biol Chem **276**(27): 25605-11.

Brummelkamp, T. R., R. Bernards, et al. (2002). "Stable suppression of tumorigenicity by virus-mediated RNA interference." Cancer Cell **2**(3): 243-7.

Brummelkamp, T. R., R. Bernards, et al. (2002). "A system for stable expression of short interfering RNAs in mammalian cells." Science **296**(5567): 550-3.

Burtey, A., J. Z. Rappoport, et al. (2007). "Dynamic interaction of HIV-1 Nef with the clathrin-mediated endocytic pathway at the plasma membrane." Traffic **8**(1): 61-76.

Capodici, J., K. Kariko, et al. (2002). "Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference." J Immunol **169**(9): 5196-201.

Carl, S., R. Daniels, et al. (2000). "Partial "repair" of defective NEF genes in a long-term nonprogressor with human immunodeficiency virus type 1 infection." J Infect Dis **181**(1): 132-40.

Chalk, A. M., C. Wahlestedt, et al. (2004). "Improved and automated prediction of effective siRNA." Biochem Biophys Res Commun **319**(1): 264-74.

Chang, L. J., X. Liu, et al. (2005). "Lentiviral siRNAs targeting multiple highly conserved RNA sequences of human immunodeficiency virus type 1." Gene Ther **12**(14): 1133-44.

Chen, Y. L., D. Trono, et al. (1998). "The proteolytic cleavage of human immunodeficiency virus type 1 Nef does not correlate with its ability to stimulate virion infectivity." J Virol **72**(4): 3178-84.

Chendrimada, T. P., R. I. Gregory, et al. (2005). "TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing." Nature **436**(7051): 740-4.

Choe, H., M. Farzan, et al. (1996). "The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates." Cell **85**(7): 1135-48.

Choi, H. J. and T. E. Smithgall (2004). "Conserved residues in the HIV-1 Nef hydrophobic pocket are essential for recruitment and activation of the Hck tyrosine kinase." J Mol Biol **343**(5): 1255-68.

Choi, I., B. R. Cho, et al. (2005). "Choice of the adequate detection time for the accurate evaluation of the efficiency of siRNA-induced gene silencing." J Biotechnol **120**(3): 251-61.

Chung, K. H., C. C. Hart, et al. (2006). "Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155." Nucleic Acids Res **34**(7): e53.

Coates, K., S. J. Cooke, et al. (1997). "Protein kinase C-mediated phosphorylation of HIV-I nef in human cell lines." J Biol Chem **272**(19): 12289-94.

Cohen, G. B., R. T. Gandhi, et al. (1999). "The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells." Immunity **10**(6): 661-71.

Collins, K. L. and D. Baltimore (1999). "HIV's evasion of the cellular immune response." Immunol Rev **168**: 65-74.

Daniel, M. D., F. Kirchhoff, et al. (1992). "Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene." Science **258**(5090): 1938-41.

Das, A. T., T. R. Brummelkamp, et al. (2004). "Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition." J Virol **78**(5): 2601-5.

Dave, R. S. and R. J. Pomerantz (2004). "Antiviral effects of human immunodeficiency virus type 1-specific small interfering RNAs against targets conserved in select neurotropic viral strains." J Virol **78**(24): 13687-96.

Davis, M. (2006). ApE- A plasmid editor.

Deacon, N. J., A. Tsykin, et al. (1995). "Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients." Science **270**(5238): 988-91.

Delassus, S., R. Cheynier, et al. (1991). "Evolution of human immunodeficiency virus type 1 nef and long terminal repeat sequences over 4 years in vivo and in vitro." J Virol **65**(1): 225-31.

Denli, A. M., B. B. Tops, et al. (2004). "Processing of primary microRNAs by the Microprocessor complex." Nature **432**(7014): 231-5.

Djordjevic, J. T., S. D. Schibeci, et al. (2004). "HIV type 1 Nef increases the association of T cell receptor (TCR)-signaling molecules with T cell rafts and promotes activation-induced raft fusion." AIDS Res Hum Retroviruses **20**(5): 547-55.

Elbashir, S. M., J. Harborth, et al. (2001). "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." Nature **411**(6836): 494-8.

Elbashir, S. M., J. Harborth, et al. (2002). "Analysis of gene function in somatic mammalian cells using small interfering RNAs." Methods **26**(2): 199-213.

Elbashir, S. M., J. Martinez, et al. (2001). "Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate." Embo J **20**(23): 6877-88.

Enose, Y., M. Kita, et al. (2004). "Protective effects of nef-deleted SHIV or that having IFN-gamma against disease induced with a pathogenic virus early after vaccination." Arch Virol **149**(9): 1705-20.

Eulalio, A., I. Behm-Ansmant, et al. (2007). "P-body formation is a consequence, not the cause, of RNA-mediated gene silencing." Mol Cell Biol **27**(11): 3970-81.

Fackler, O. T., N. Kienzle, et al. (1997). "Association of human immunodeficiency virus Nef protein with actin is myristoylation dependent and influences its subcellular localization." Eur J Biochem **247**(3): 843-51.

Fackler, O. T., A. Moris, et al. (2006). "Functional characterization of HIV-1 Nef mutants in the context of viral infection." Virology **351**(2): 322-39.

Fenard, D., W. Yonemoto, et al. (2005). "Nef is physically recruited into the immunological synapse and potentiates T cell activation early after TCR engagement." J Immunol **175**(9): 6050-7.

Fire, A., D. Albertson, et al. (1991). "Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle." Development **113**(2): 503-14.

Fire, A., S. Xu, et al. (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-11.

Franchini, G., M. Robert-Guroff, et al. (1986). "Cytoplasmic localization of the HTLV-III 3' orf protein in cultured T cells." Virology **155**(2): 593-9.

Frankel, A. D. and J. A. Young (1998). "HIV-1: fifteen proteins and an RNA." Annu Rev Biochem **67**: 1-25.

Freund, J., R. Kellner, et al. (1994). "Stability and proteolytic domains of Nef protein from human immunodeficiency virus (HIV) type 1." Eur J Biochem **221**(2): 811-9.

Garcia, J. V., J. Alfano, et al. (1993). "The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4." J Virol **67**(3): 1511-6.

Gaynor, R. (1992). "Cellular transcription factors involved in the regulation of HIV-1 gene expression." Aids **6**(4): 347-63.

Geyer, M., C. E. Munte, et al. (1999). "Structure of the anchor-domain of myristoylated and non-myristoylated HIV-1 Nef protein." J Mol Biol **289**(1): 123-38.

Geyer, M. and B. M. Peterlin (2001). "Domain assembly, surface accessibility and sequence conservation in full length HIV-1 Nef." FEBS Lett **496**(2-3): 91-5.

Giolo, G., F. Neri, et al. (2007). "Internalization and intracellular retention of CD4 are two separate functions of the human immunodeficiency virus type 1 Nef protein." J Gen Virol **88**(Pt 11): 3133-8.

Glushakova, S., J. Munch, et al. (2001). "CD4 down-modulation by human immunodeficiency virus type 1 Nef correlates with the efficiency of viral replication and with CD4(+) T-cell depletion in human lymphoid tissue ex vivo." J Virol **75**(21): 10113-7.

Gombert, F. O., W. Blecha, et al. (1990). "Antigenic epitopes of NEF proteins from different HIV-1 strains as recognized by sera from patients with manifest and latent HIV infection." Virology **176**(2): 458-66.

Gou, D., T. Weng, et al. (2007). "A novel approach for the construction of multiple shRNA expression vectors." J Gene Med **9**(9): 751-63.

Greenberg, M. E., S. Bronson, et al. (1997). "Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation." Embo J **16**(23): 6964-76.

Greenberg, M. E., A. J. Iafrate, et al. (1998). "The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes." Embo J **17**(10): 2777-89.

Greene, W. C. and B. M. Peterlin (2002). "Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy." Nat Med **8**(7): 673-80.

Greenway, A., A. Azad, et al. (1995). "Human immunodeficiency virus type 1 Nef protein inhibits activation pathways in peripheral blood mononuclear cells and T-cell lines." J Virol **69**(3): 1842-50.

Greenway, A. L., D. A. McPhee, et al. (1994). "Nef 27, but not the Nef 25 isoform of human immunodeficiency virus-type 1 pNL4.3 down-regulates surface CD4 and IL-2R expression in peripheral blood mononuclear cells and transformed T cells." Virology **198**(1): 245-56.

Grimm, D., K. L. Streetz, et al. (2006). "Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways." Nature **441**(7092): 537-41.

Grzesiek, S., A. Bax, et al. (1997). "Refined solution structure and backbone dynamics of HIV-1 Nef." Protein Sci **6**(6): 1248-63.

Grzesiek, S., S. J. Stahl, et al. (1996). "The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR." Biochemistry **35**(32): 10256-61.

Guy, B., M. P. Kieny, et al. (1987). "HIV F/3' orf encodes a phosphorylated GTP-binding protein resembling an oncogene product." Nature **330**(6145): 266-9.

Haase, A. D., L. Jaskiewicz, et al. (2005). "TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing." EMBO Rep **6**(10): 961-7.

Hamilton, A. J. and D. C. Baulcombe (1999). "A species of small antisense RNA in posttranscriptional gene silencing in plants." Science **286**(5441): 950-2.

Hammond, S. M. (2005). "Dicing and slicing: the core machinery of the RNA interference pathway." FEBS Lett **579**(26): 5822-9.

Han, J., Y. Lee, et al. (2004). "The Drosha-DGCR8 complex in primary microRNA processing." Genes Dev **18**(24): 3016-27.

Han, J., Y. Lee, et al. (2006). "Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex." Cell **125**(5): 887-901.

Heale, B. S., H. S. Soifer, et al. (2005). "siRNA target site secondary structure predictions using local stable substructures." Nucleic Acids Res **33**(3): e30.

Heeney, J. L., A. G. Dalgleish, et al. (2006). "Origins of HIV and the evolution of resistance to AIDS." Science **313**(5786): 462-6.

Heinonen, J. E., A. J. Mohamed, et al. (2005). "Inducible H1 promoter-driven lentiviral siRNA expression by Stuffer reporter deletion." Oligonucleotides **15**(2): 139-44.

Hillis, D. M. (2000). "AIDS. Origins of HIV." Science **288**(5472): 1757-9.

Hohjoh, H. (2002). "RNA interference (RNA(i)) induction with various types of synthetic oligonucleotide duplexes in cultured human cells." FEBS Lett **521**(1-3): 195-9.

Huang, Y., L. Zhang, et al. (1995). "Characterization of nef sequences in long-term survivors of human immunodeficiency virus type 1 infection." J Virol **69**(1): 93-100.

Hung, C. H., L. Thomas, et al. (2007). "HIV-1 Nef assembles a Src family kinase-ZAP-70/Syk-PI3K cascade to downregulate cell-surface MHC-I." Cell Host Microbe **1**(2): 121-33.

Hutvagner, G. and P. D. Zamore (2002). "A microRNA in a multiple-turnover RNAi enzyme complex." Science **297**(5589): 2056-60.

Jackson, A. L., S. R. Bartz, et al. (2003). "Expression profiling reveals off-target gene regulation by RNAi." Nat Biotechnol **21**(6): 635-7.

Jackson, A. L., J. Burchard, et al. (2006). "Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing." Rna **12**(7): 1197-205.

Jacque, J. M., K. Triques, et al. (2002). "Modulation of HIV-1 replication by RNA interference." Nature **418**(6896): 435-8.

Jagla, B., N. Aulner, et al. (2005). "Sequence characteristics of functional siRNAs." Rna **11**(6): 864-72.

Jin, Y. J., C. Y. Cai, et al. (2005). "HIV Nef-mediated CD4 down-regulation is adaptor protein complex 2 dependent." J Immunol **175**(5): 3157-64.

Johnson, D. C. and M. T. Huber (2002). "Directed egress of animal viruses promotes cell-to-cell spread." J Virol **76**(1): 1-8.

Judge, A. D., G. Bola, et al. (2006). "Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo." Mol Ther **13**(3): 494-505.

Judge, A. D., V. Sood, et al. (2005). "Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA." Nat Biotechnol **23**(4): 457-62.

Kaminchik, J., N. Bashan, et al. (1991). "Genetic characterization of human immunodeficiency virus type 1 nef gene products translated in vitro and expressed in mammalian cells." J Virol **65**(2): 583-8.

Kaplan, A. H., M. Manchester, et al. (1994). "The activity of the protease of human immunodeficiency virus type 1 is initiated at the membrane of infected cells before the release of viral proteins and is required for release to occur with maximum efficiency." J Virol **68**(10): 6782-6.

Kestler, H. W., 3rd, D. J. Ringler, et al. (1991). "Importance of the nef gene for maintenance of high virus loads and for development of AIDS." Cell **65**(4): 651-62.

Kienzle, N., J. Freund, et al. (1993). "Oligomerization of the Nef protein from human immunodeficiency virus (HIV) type 1." Eur J Biochem **214**(2): 451-7.

Kirchhoff, F., P. J. Easterbrook, et al. (1999). "Sequence variations in human immunodeficiency virus type 1 Nef are associated with different stages of disease." J Virol **73**(7): 5497-508.

Kok, K. H., M. H. Ng, et al. (2007). "Human TRBP and PACT directly interact with each other and associate with dicer to facilitate the production of small interfering RNA." J Biol Chem **282**(24): 17649-57.

Konstantinova, P., W. de Vries, et al. (2006). "Inhibition of human immunodeficiency virus type 1 by RNA interference using long-hairpin RNA." Gene Ther **13**(19): 1403-13.

Korber, B., M. Muldoon, et al. (2000). "Timing the ancestor of the HIV-1 pandemic strains." Science **288**(5472): 1789-96.

Laguette, N., S. Benichou, et al. (2009). "Human immunodeficiency virus type 1 Nef incorporation into virions does not increase infectivity." J Virol **83**(2): 1093-104.

Laurent, A. G., A. G. Hovanessian, et al. (1990). "Production of a non-functional nef protein in human immunodeficiency virus type 1-infected CEM cells." J Gen Virol **71 (Pt 10)**: 2273-81.

Le Gall, S., L. Erdtmann, et al. (1998). "Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules." Immunity **8**(4): 483-95.

Learmont, J. C., A. F. Geczy, et al. (1999). "Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort." N Engl J Med **340**(22): 1715-22.

Lee, C. H., K. Saksela, et al. (1996). "Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain." Cell **85**(6): 931-42.

Lee, R. C., R. L. Feinbaum, et al. (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**(5): 843-54.

Lee, Y., C. Ahn, et al. (2003). "The nuclear RNase III Drosha initiates microRNA processing." Nature **425**(6956): 415-9.

Lee, Y., I. Hur, et al. (2006). "The role of PACT in the RNA silencing pathway." Embo J **25**(3): 522-32.

Lee, Y., K. Jeon, et al. (2002). "MicroRNA maturation: stepwise processing and subcellular localization." Embo J **21**(17): 4663-70.

Lee, Y., M. Kim, et al. (2004). "MicroRNA genes are transcribed by RNA polymerase II." Embo J **23**(20): 4051-60.

Leirdal, M. and M. Sioud (2002). "Gene silencing in mammalian cells by preformed small RNA duplexes." Biochem Biophys Res Commun **295**(3): 744-8.

Leitner, T. (2006). HIV Sequence Compedium 2006/2007. Los Alamos, Los Alamos National Laboratory.

Li, M. J., G. Bauer, et al. (2003). "Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs." Mol Ther **8**(2): 196-206.

Li, P. L., T. Wang, et al. (2005). "Phosphorylation of HIV Nef by cAMP-dependent protein kinase." Virology **331**(2): 367-74.

Li, X., X. Zhao, et al. (1998). "Generation of destabilized green fluorescent protein as a transcription reporter." J Biol Chem **273**(52): 34970-5.

Lindwasser, O. W., W. J. Smith, et al. (2008). "A diacidic motif in human immunodeficiency virus type 1 Nef is a novel determinant of binding to AP-2." J Virol **82**(3): 1166-74.

Liu, J., M. A. Carmell, et al. (2004). "Argonaute2 is the catalytic engine of mammalian RNAi." Science **305**(5689): 1437-41.

Liu, Y. P., J. Haasnoot, et al. (2008). "Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron." Nucleic Acids Res **36**(9): 2811-24.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-8.

Luciw, P. A., C. Cheng-Mayer, et al. (1987). "Mutational analysis of the human immunodeficiency virus: the orf-B region down-regulates virus replication." Proc Natl Acad Sci U S A **84**(5): 1434-8.

Lund, E., S. Guttinger, et al. (2004). "Nuclear export of microRNA precursors." Science **303**(5654): 95-8.

Mangasarian, A., M. Foti, et al. (1997). "The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane." Immunity **6**(1): 67-77.

Mariani, R., F. Kirchhoff, et al. (1996). "High frequency of defective nef alleles in a long-term survivor with nonprogressive human immunodeficiency virus type 1 infection." J Virol **70**(11): 7752-64.

Marshall, O. J. (2004). "PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR." Bioinformatics **20**(15): 2471-2.

Martinez, J., A. Patkaniowska, et al. (2002). "Single-stranded antisense siRNAs guide target RNA cleavage in RNAi." Cell **110**(5): 563-74.

McWilliams, M. J., J. G. Julias, et al. (2003). "Mutations in the 5' end of the human immunodeficiency virus type 1 polypurine tract affect RNase H cleavage specificity and virus titer." J Virol **77**(20): 11150-7.

Mendell, J. T. (2005). "MicroRNAs: critical regulators of development, cellular physiology and malignancy." Cell Cycle **4**(9): 1179-84.

Miller, A. D. and G. J. Rosman (1989). "Improved retroviral vectors for gene transfer and expression." Biotechniques **7**(9): 980-2, 984-6, 989-90.

Miller, M. D., M. T. Warmerdam, et al. (1994). "The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages." J Exp Med **179**(1): 101-13.

Miyauchi, K., Y. Kim, et al. (2009). "HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes." Cell **137**(3): 433-44.

Moarefi, I., M. LaFevre-Bernt, et al. (1997). "Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement." Nature **385**(6617): 650-3.

Munch, J., D. Rajan, et al. (2005). "The role of upstream U3 sequences in HIV-1 replication and CD4+ T cell depletion in human lymphoid tissue ex vivo." Virology **341**(2): 313-20.

Munch, J., D. Rajan, et al. (2007). "Nef-mediated enhancement of virion infectivity and stimulation of viral replication are fundamental properties of primate lentiviruses." J Virol **81**(24): 13852-64.

Murphy, K. M., M. J. Sweet, et al. (1993). "Effects of the tat and nef gene products of human immunodeficiency virus type 1 (HIV-1) on transcription controlled by the HIV-1 long terminal repeat and on cell growth in macrophages." J Virol **67**(12): 6956-64.

Niederman, T. M., W. R. Hastings, et al. (1993). "Myristoylation-enhanced binding of the HIV-1 Nef protein to T cell skeletal matrix." Virology **197**(1): 420-5.

Niederman, T. M., B. J. Thielan, et al. (1989). "Human immunodeficiency virus type 1 negative factor is a transcriptional silencer." Proc Natl Acad Sci U S A **86**(4): 1128-32.

Novina, C. D., M. F. Murray, et al. (2002). "siRNA-directed inhibition of HIV-1 infection." Nat Med **8**(7): 681-6.

Omoto, S. and Y. R. Fujii (2005). "Regulation of human immunodeficiency virus 1 transcription by nef microRNA." J Gen Virol **86**(Pt 3): 751-5.

Omoto, S., M. Ito, et al. (2004). "HIV-1 nef suppression by virally encoded microRNA." Retrovirology **1**: 44.

O'Neill, E., L. S. Kuo, et al. (2006). "Dynamic evolution of the human immunodeficiency virus type 1 pathogenic factor, Nef." J Virol **80**(3): 1311-20.

Otake, K., Y. Fujii, et al. (1997). "Epitope mapping of murine monoclonal antibodies against human immunodeficiency virus type 1 Nef." Exp Anim **46**(1): 53-8.

Paddison, P. J., A. A. Caudy, et al. (2002). "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." Genes Dev **16**(8): 948-58.

Pancoska, P., Z. Moravek, et al. (2004). "Rational design of DNA sequences for nanotechnology, microarrays and molecular computers using Eulerian graphs." Nucleic Acids Res **32**(15): 4630-45.

Pandori, M., H. Craig, et al. (1998). "Virological importance of the protease-cleavage site in human immunodeficiency virus type 1 Nef is independent of both intravirion processing and CD4 down-regulation." Virology **251**(2): 302-16.

Park, W. S., N. Miyano-Kurosaki, et al. (2002). "Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference." Nucleic Acids Res **30**(22): 4830-5.

Paul, C. P., P. D. Good, et al. (2002). "Effective expression of small interfering RNA in human cells." Nat Biotechnol **20**(5): 505-8.

Peng, B. and M. Robert-Guroff (2001). "Deletion of N-terminal myristoylation site of HIV Nef abrogates both MHC-1 and CD4 down-regulation." Immunol Lett **78**(3): 195-200.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.

Piguet, V., F. Gu, et al. (1999). "Nef-induced CD4 degradation: a diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of beta-COP in endosomes." Cell **97**(1): 63-73.

Powell, M. D. and J. G. Levin (1996). "Sequence and structural determinants required for priming of plus-strand DNA synthesis by the human immunodeficiency virus type 1 polypurine tract." J Virol **70**(8): 5288-96.

Pullen, K. A., A. J. Rattray, et al. (1993). "The sequence features important for plus strand priming by human immunodeficiency virus type 1 reverse transcriptase." J Biol Chem **268**(9): 6221-7.

Qiu, S., C. M. Adema, et al. (2005). "A computational study of off-target effects of RNA interference." Nucleic Acids Res **33**(6): 1834-47.

Ramakers, C., J. M. Ruijter, et al. (2003). "Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data." Neurosci Lett **339**(1): 62-6.

Ranki, A., A. Lagerstedt, et al. (1994). "Expression kinetics and subcellular localization of HIV-1 regulatory proteins Nef, Tat and Rev in acutely and chronically infected lymphoid cell lines." Arch Virol **139**(3-4): 365-78.

Ratner, L., A. Fisher, et al. (1987). "Complete nucleotide sequences of functional clones of the AIDS virus." AIDS Res Hum Retroviruses **3**(1): 57-69.

Rausch, J. W. and S. F. Le Grice (2004). "'Binding, bending and bonding': polypurine tract-primed initiation of plus-strand DNA synthesis in human immunodeficiency virus." Int J Biochem Cell Biol **36**(9): 1752-66.

Reynolds, A., D. Leake, et al. (2004). "Rational siRNA design for RNA interference." Nat Biotechnol **22**(3): 326-30.

Robert-Guroff, M., M. Popovic, et al. (1990). "Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages." J Virol **64**(7): 3391-8.

Roeth, J. F., M. Williams, et al. (2004). "HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail." J Cell Biol **167**(5): 903-13.

Rose, J. J., K. Janvier, et al. (2005). "CD4 down-regulation by HIV-1 and simian immunodeficiency virus (SIV) Nef proteins involves both internalization and intracellular retention mechanisms." J Biol Chem **280**(9): 7413-26.

Ruijter, J. M., C. Ramakers, et al. (2009). "Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data." Nucleic Acids Res **37**(6): e45.

Rutjens, E., S. Balla-Jhagjhoorsingh, et al. (2003). "Lentivirus infections and mechanisms of disease resistance in chimpanzees." Front Biosci **8**: d1134-45.

Saayman, S., S. Barichievy, et al. (2008). "The efficacy of generating three independent anti-HIV-1 siRNAs from a single U6 RNA Pol III-expressed long hairpin RNA." PLoS One **3**(7): e2602.

Saksela, K. (1997). "HIV-1 Nef and host cell protein kinases." Front Biosci **2**: d606-18.

Saksela, K., G. Cheng, et al. (1995). "Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4." Embo J **14**(3): 484-91.

Saxena, S., Z. O. Jonsson, et al. (2003). "Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells." J Biol Chem **278**(45): 44312-9.

Schaefer, M. R., E. R. Wonderlich, et al. (2008). "HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells." PLoS Pathog **4**(8): e1000131.

Schefe, J. H., K. E. Lehmann, et al. (2006). "Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula." J Mol Med **84**(11): 901-10.

Schubert, S., A. Grunweller, et al. (2005). "Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions." J Mol Biol **348**(4): 883-93.

Schwarz, D. S., G. Hutvagner, et al. (2003). "Asymmetry in the assembly of the RNAi enzyme complex." Cell **115**(2): 199-208.

Seibel, N. M., J. Eljouni, et al. (2007). "Nuclear localization of enhanced green fluorescent protein homomultimers." Anal Biochem **368**(1): 95-9.

Semizarov, D., L. Frost, et al. (2003). "Specificity of short interfering RNA determined through gene expression signatures." Proc Natl Acad Sci U S A **100**(11): 6347-52.

Shin, K. J., E. A. Wall, et al. (2006). "A single lentiviral vector platform for microRNA-based conditional RNA interference and coordinated transgene expression." Proc Natl Acad Sci U S A **103**(37): 13759-64.

Shugars, D. C., M. S. Smith, et al. (1993). "Analysis of human immunodeficiency virus type 1 nef gene sequences present in vivo." J Virol **67**(8): 4639-50.

Simmons, A., V. Aluvihare, et al. (2001). "Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators." Immunity **14**(6): 763-77.

Siolas, D., C. Lerner, et al. (2005). "Synthetic shRNAs as potent RNAi triggers." Nat Biotechnol **23**(2): 227-31.

Sledz, C. A., M. Holko, et al. (2003). "Activation of the interferon system by short-interfering RNAs." Nat Cell Biol **5**(9): 834-9.

Song, J. J., S. K. Smith, et al. (2004). "Crystal structure of Argonaute and its implications for RISC slicer activity." Science **305**(5689): 1434-7.

Stowell, D. (2002). 3D HIV Interactive 3D model.

Taxman, D. J., L. R. Livingstone, et al. (2006). "Criteria for effective design, construction, and gene knockdown by shRNA vectors." BMC Biotechnol **6**: 7.

Taylor, B. S. and S. M. Hammer (2008). "The challenge of HIV-1 subtype diversity." N Engl J Med **359**(18): 1965-6.

ten Haaft, P., K. Murthy, et al. (2001). "Differences in early virus loads with different phenotypic variants of HIV-1 and SIV(cpz) in chimpanzees." Aids **15**(16): 2085-92.

ter Brake, O. and B. Berkhout (2005). "A novel approach for inhibition of HIV-1 by RNA interference: counteracting viral escape with a second generation of siRNAs." Journal of RNAi and Gene Silencing **1**(2): 56-65.

ter Brake, O., P. Konstantinova, et al. (2006). "Silencing of HIV-1 with RNA interference: a multiple shRNA approach." Mol Ther **14**(6): 883-92.

Terwilliger, E., J. G. Sodroski, et al. (1986). "Effects of mutations within the 3' orf open reading frame region of human T-cell lymphotropic virus type III (HTLV-III/LAV) on replication and cytopathogenicity." J Virol **60**(2): 754-60.

Thoulouze, M. I., N. Sol-Foulon, et al. (2006). "Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse." Immunity **24**(5): 547-61.

Ui-Tei, K., Y. Naito, et al. (2008). "Thermodynamic stability and Watson-Crick base pairing in the seed duplex are major determinants of the efficiency of the siRNA-based off-target effect." Nucleic Acids Res **36**(22): 7100-9.

Ui-Tei, K., Y. Naito, et al. (2006). "Essential Notes Regarding the Design of Functional siRNAs for Efficient Mammalian RNAi." J Biomed Biotechnol **2006**(4): 65052.

Ui-Tei, K., Y. Naito, et al. (2004). "Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference." Nucleic Acids Res **32**(3): 936-48.

UNAIDS (2008). Report on the global AIDS epidemic. Executive summary. Switzerland, UNAIDS.

van der Krol, A. R., L. A. Mur, et al. (1990). "Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression." Plant Cell **2**(4): 291-9.

Vanhecke, D. and M. Janitz (2005). "Functional genomics using high-throughput RNA interference." Drug Discov Today **10**(3): 205-12.

Vickers, T. A., W. F. Lima, et al. (2007). "Reduced levels of Ago2 expression result in increased siRNA competition in mammalian cells." Nucleic Acids Res **35**(19): 6598-610.

Wang, L. and F. Y. Mu (2004). "A Web-based design center for vector-based siRNA and siRNA cassette." Bioinformatics **20**(11): 1818-20.

Watson, S., S. Mercier, et al. (2007). "Determination of suitable housekeeping genes for normalisation of quantitative real time PCR analysis of cells infected with human immunodeficiency virus and herpes viruses." Virol J **4**: 130.

Weiss, R. A. (1993). "How does HIV cause AIDS?" Science **260**(5112): 1273-9.

Welker, R., M. Harris, et al. (1998). "Virion incorporation of human immunodeficiency virus type 1 Nef is mediated by a bipartite membrane-targeting signal: analysis of its role in enhancement of viral infectivity." J Virol **72**(11): 8833-40.

Welker, R., H. Kottler, et al. (1996). "Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase." Virology **219**(1): 228-36.

Westerhout, E. M., M. Ooms, et al. (2005). "HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome." Nucleic Acids Res **33**(2): 796-804.

Westerhout, E. M., O. ter Brake, et al. (2006). "The virion-associated incoming HIV-1 RNA genome is not targeted by RNA interference." Retrovirology **3**: 57.

Wolf, D., S. I. Giese, et al. (2008). "Novel (n)PKC kinases phosphorylate Nef for increased HIV transcription, replication and perinuclear targeting." Virology **370**(1): 45-54.

Wu, Y. and J. W. Marsh (2001). "Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA." Science **293**(5534): 1503-6.

Wyatt, R. and J. Sodroski (1998). "The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens." Science **280**(5371): 1884-8.

Yamamoto, T., H. Miyoshi, et al. (2006). "Lentivirus vectors expressing short hairpin RNAs against the U3-overlapping region of HIV nef inhibit HIV replication and infectivity in primary macrophages." Blood **108**(10): 3305-12.

Yamamoto, T., S. Omoto, et al. (2002). "Double-stranded nef RNA interferes with human immunodeficiency virus type 1 replication." Microbiol Immunol **46**(11): 809-17.

Ye, H., H. J. Choi, et al. (2004). "Oligomerization is required for HIV-1 Nef-induced activation of the Src family protein-tyrosine kinase, Hck." Biochemistry **43**(50): 15775-84.

Yoon, K., J. G. Jeong, et al. (2001). "Differential effects of primary human immunodeficiency virus type 1 nef sequences on downregulation of CD4 and MHC class I." Biochem Biophys Res Commun **284**(3): 638-42.

Yu, J. Y., S. L. DeRuiter, et al. (2002). "RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells." Proc Natl Acad Sci U S A **99**(9): 6047-52.

Yuan, B., R. Latek, et al. (2004). "siRNA Selection Server: an automated siRNA oligonucleotide prediction server." Nucleic Acids Res **32**(Web Server issue): W130-4.

Zamore, P. D. and B. Haley (2005). "Ribo-gnome: the big world of small RNAs." Science **309**(5740): 1519-24.

Zazopoulos, E. and W. A. Haseltine (1993). "Effect of nef alleles on replication of human immunodeficiency virus type 1." Virology **194**(1): 20-7.

Zeng, Y. and B. R. Cullen (2003). "Sequence requirements for micro RNA processing and function in human cells." Rna **9**(1): 112-23.

Zeng, Y., E. J. Wagner, et al. (2002). "Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells." Mol Cell **9**(6): 1327-33.

Zeng, Y., R. Yi, et al. (2003). "MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms." Proc Natl Acad Sci U S A **100**(17): 9779-84.

Zhang, H., F. A. Kolb, et al. (2004). "Single processing center models for human Dicer and bacterial RNase III." Cell **118**(1): 57-68.